

other hand, a number of high-affinity EGCG-binding proteins, including IGF-1R, have been revealed by recent affinity chromatography studies [66, 102, 103]. These studies indicate that EGCG does in fact bind to target proteins at low concentrations, although relatively high concentrations are required to exert its physiological functions. Moreover, for consideration of GTCs in the clinical practice, it should be emphasized that EGCG can inhibit the activation of EGFR at low micromolar concentrations [42, 43, 85] that are considered within the physiologically relevant range for human exposure [104]. Furthermore, EGCG preferentially inhibits the growth of cancer cells without affecting the growth of the corresponding normal cells [16, 42, 50, 105].

A possible explanation for these phenomena is the concept of “oncogene addiction” according to which cancers associated with multiple genetic, epigenetic, and chromosomal abnormalities are usually dependent on or “addicted” to one or a few genes for both maintenance of the malignant phenotype and cell survival and, therefore, targeting only one or a few of these aberrant molecules might be effective to inhibit carcinogenesis and growth of cancer cell [106, 107]. It is likely that EGCG preferentially inhibits growth and induces apoptosis in cancer cells by blocking the activity of one or a few of “addicted” oncogenic factors, including abnormalities in RTKs.

Tea is currently considered one of the most promising dietary agents for the prevention and treatment of many diseases, especially cancer. The present review provides evidence that the effects of GTCs on the inhibition of carcinogenesis are mediated, at least in part, by the regulation of the activity of certain RTKs and their related intracellular signaling pathways; this observation does not exclude other mechanisms that may also play critical roles in mediating the anticancer and cancer chemopreventive effects of these agents [1, 2]. The safety and efficacy of GTCs demonstrated in recent intervention studies [12, 93] could be crucial for the clinical application of GTCs as chemopreventive agents.

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Chemoprevention of hepatocellular carcinoma by acyclic retinoid

Masahito Shimizu, Hiroyasu Sakai, Hisataka Moriwaki

Department of Medicine, Gifu University Graduate School of Medicine, Gifu, Japan 501-1194

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11. References

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 RXR α 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 RXR α 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,

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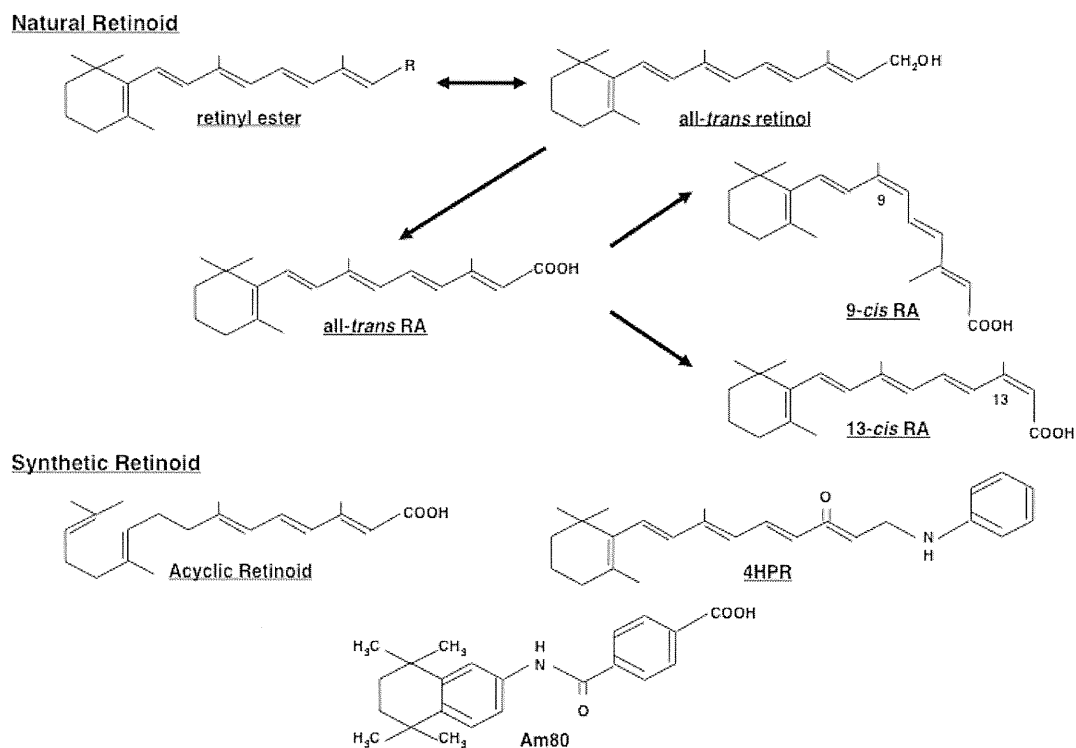


Figure 1. Chemical structures of natural and representative synthetic retinoids. Retinyl esters (mainly retinyl palmitate, R: fatty acid), stored in the liver stellate cells, are hydrolyzed to retinol. Retinoic acid (RA) is biosynthesized from retinol via the intermediate metabolite retinal by oxidation 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 (RXR α) 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 RXR α (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 RXR α 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 RXR α phosphorylation and induction of RAR β 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

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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 RXR α and RAR β , are strongly associated with the development of various human malignancies including HCC. For instance, the RAR β 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 RAR β protein and mRNA levels are also decreased in HCC (25). These findings are interesting because among the retinoid receptors, RAR β is thought to be one of the most important receptors in the regulation of cell growth and apoptosis (26).

The expression of RXR α 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 RXR α 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 RXR α (p-RXR α) (27). Specifically, RXR α 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 RXR α , 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 RXR α in a ligand-dependent manner (9, 11). In addition, although RXR α is unphosphorylated and highly ubiquitinated in a normal liver, rendering it sensitive to proteasome-mediated degradation, p-RXR α is resistant to ubiquitination and proteasome-mediated degradation in both human HCC tissues and a human HCC cell line (28). Furthermore, the phosphorylation of RXR α abolishes its ability to form heterodimers with RAR β , and this is associated with uncontrolled cell growth and resistance to

retinoids (29). These findings suggest that the accumulation of p-RXR α , (*i.e.*, non-functional RXR α) may interfere with the function of normal RXR α 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 RXR α in the negative modulation of its heterodimeric binding partners (30-32). Therefore, the inhibition of RXR α 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 RAR β expression (13, 36, 39-44). In human HCC and squamous carcinoma cells, ACR causes cell cycle arrest in G₀-G₁, 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 RAR β 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 RXR α , thus contributing to the development of HCC (9, 27). In addition, ACR also restores RXR α function by inactivating the Ras-MAPK signaling system, leading to the dephosphorylation of RXR α , although 9-*cis* RA failed to suppress ERK and RXR α phosphorylation (11). Therefore, ACR, which targets the RTK-Ras-MAPK signaling pathway and RXR α phosphorylation, is a promising agent for the chemoprevention of HCC. The role of RXR α 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

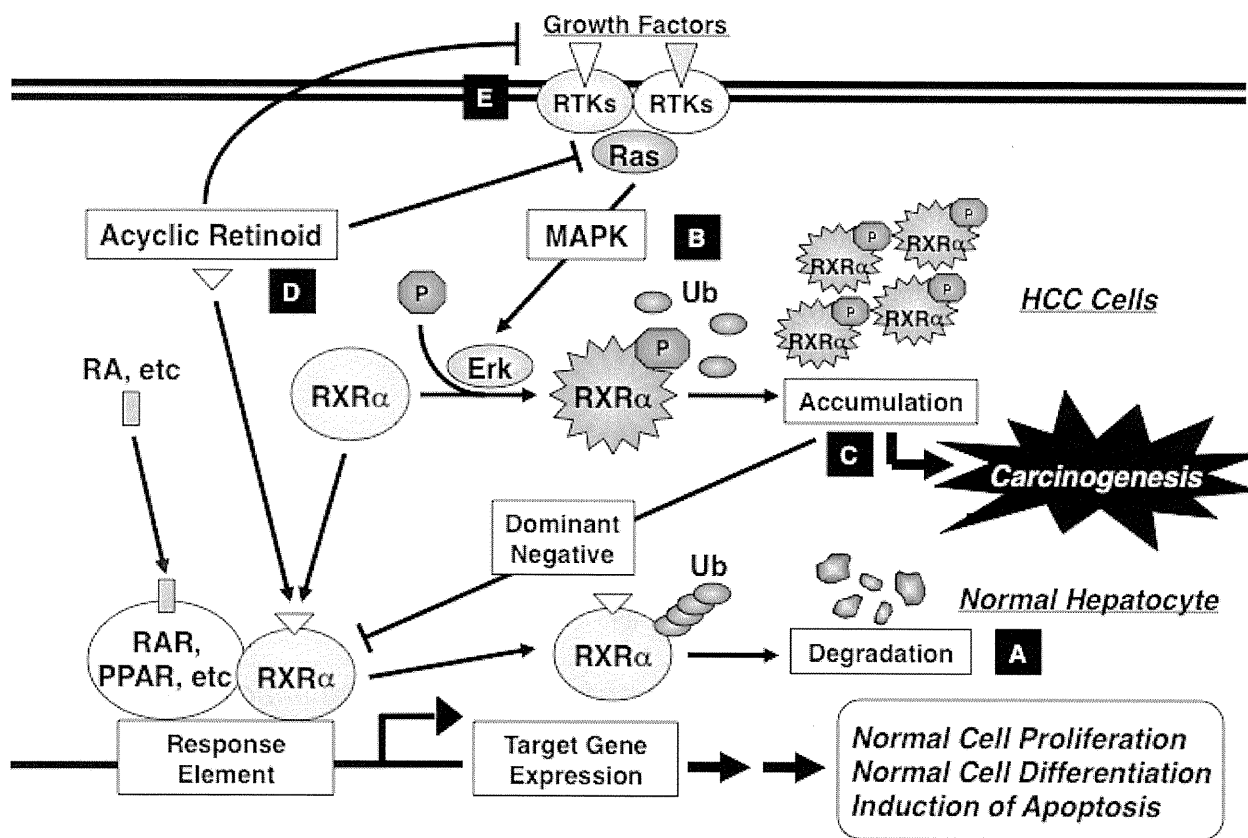


Figure 2. Retinoid-refractoriness due to phosphorylation of RXR α and its restoration by ACR in liver carcinogenesis. In normal hepatocytes, when ACR binds to and activates RXR α , it forms homo- and/or heterodimers with other nuclear receptors including RARs and PPARs, and then activates the expression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

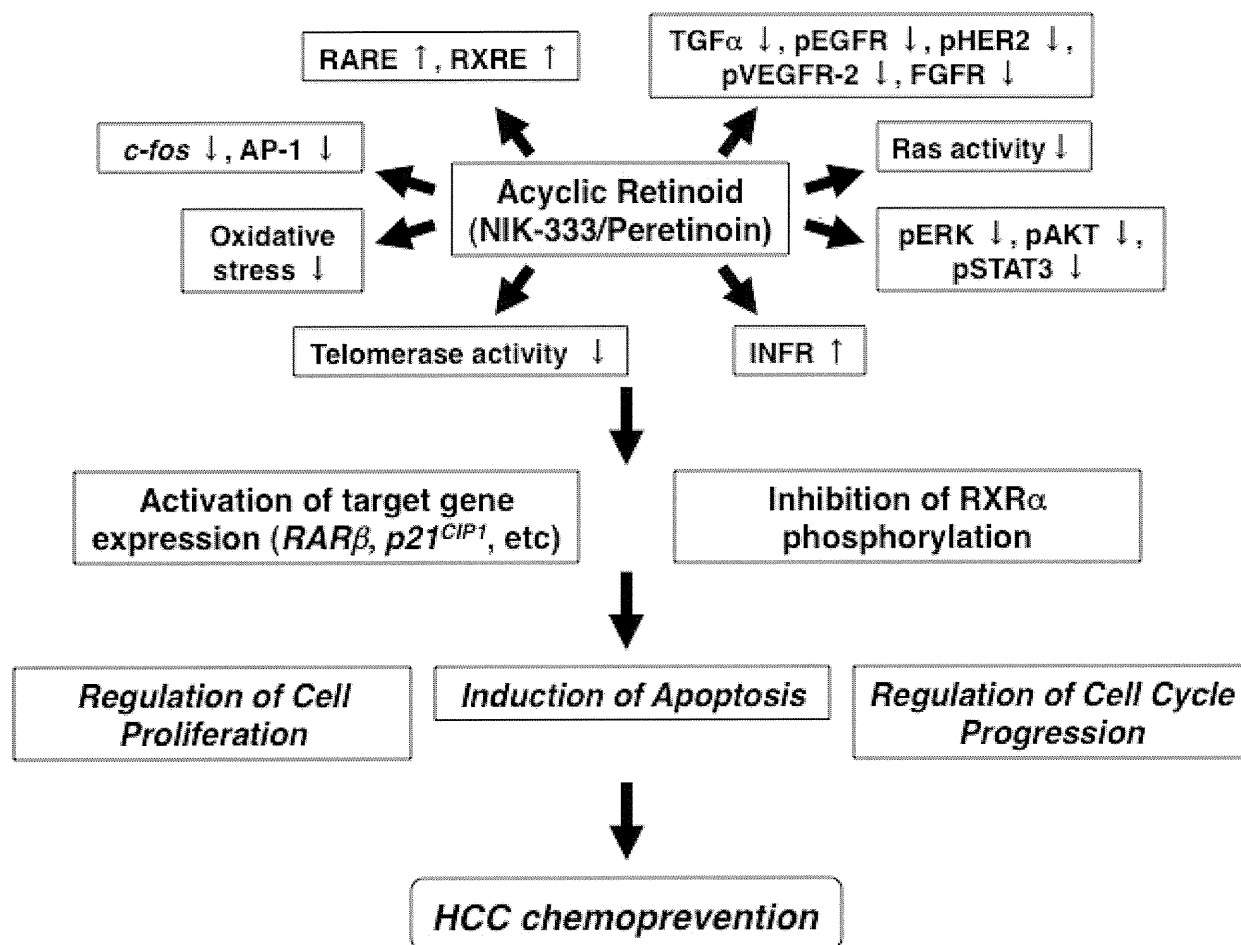


Figure 3. Pleiotropic effects of ACR to prevent HCC development. One of the main effects of ACR is to activate the expression of its target genes, such as *RARβ* and *p21^{CIP1}*, by upregulating the promoter activity of RARE and RXRE. In addition, ACR suppresses cancer cell growth by inhibiting activation 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-

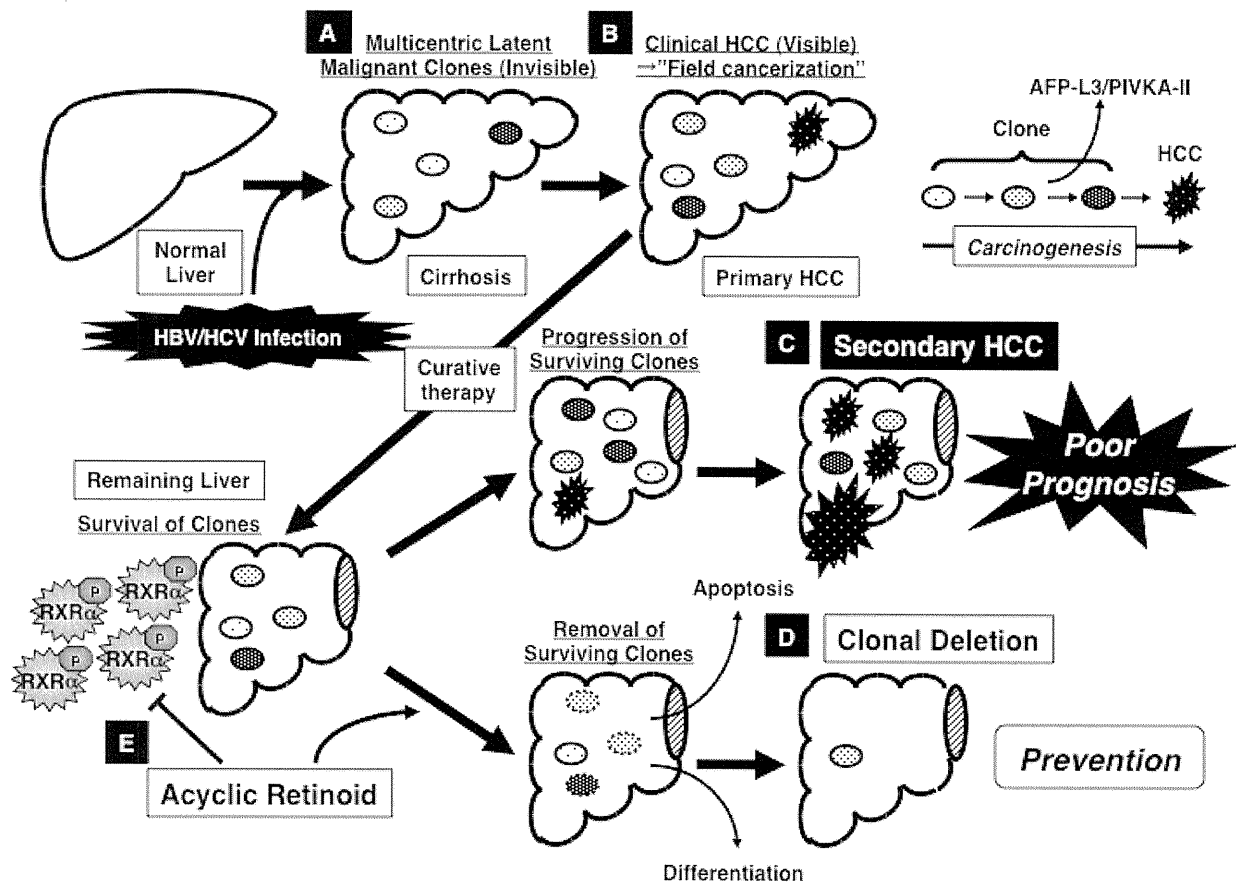


Figure 4. The concept of “clonal deletion” therapy for HCC chemoprevention. Persistent inflammation caused by hepatitis viral infection transforms the liver into a “pre-cancerous field”, which consists of multiple latent malignant clones that arise through multicentric carcinogenesis and are clinically 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 RXR α (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 K₂ (VK₂) synergistically inhibits cell growth and induces apoptosis in HCC cells

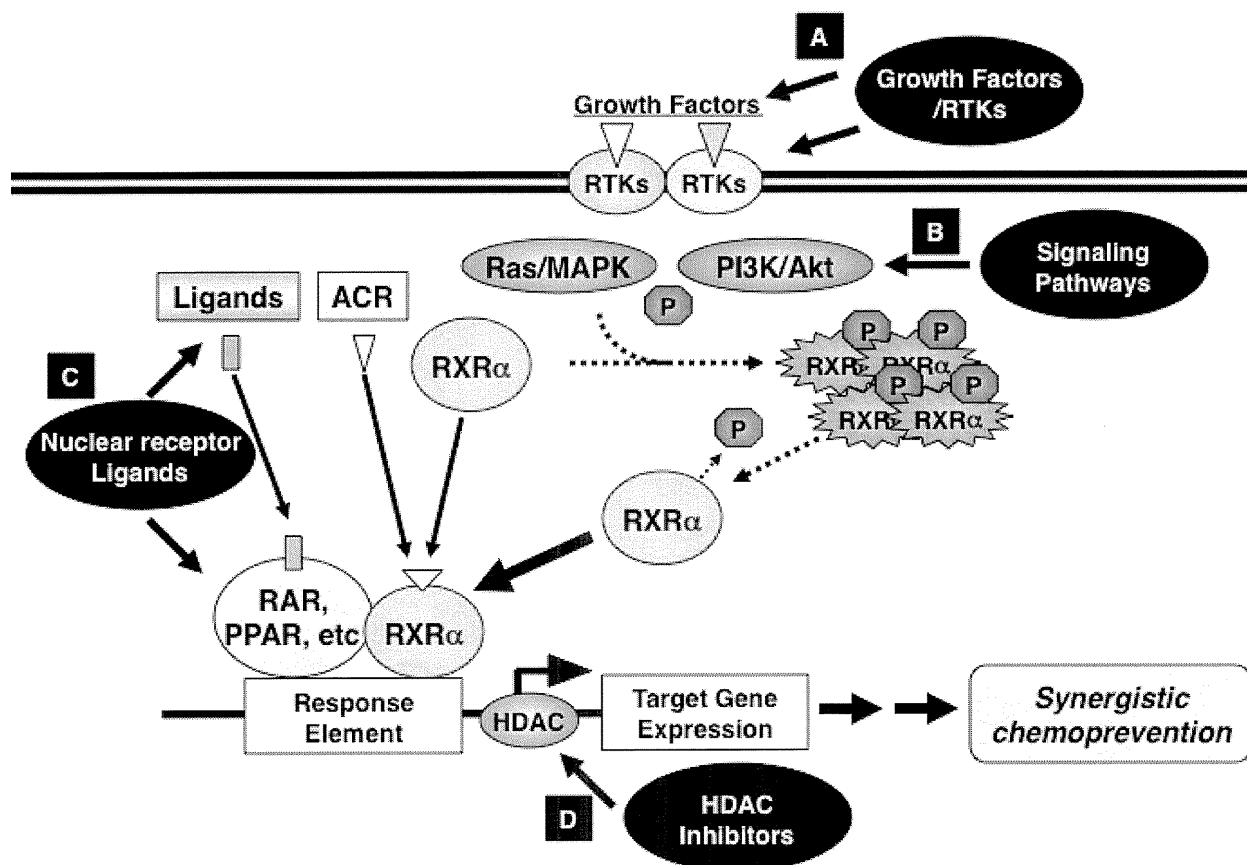


Figure 5. The possibility of “combination chemoprevention” for HCC using ACR as the key agent. Dephosphorylation of RXR α and subsequent restoration of the function of this nuclear receptor are critical to prevent the development of HCC. Therefore, the agents which target growth factor and their corresponding RTKs (A), as well as their related signaling pathways (B), including the Ras-MAPK and PI3K-Akt signaling pathways that phosphorylate RXR α , 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 VK₂ 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 RXR α . Indeed, ACR and VK₂ cooperatively inhibit activation of the Ras-MAPK signaling pathway, thus suppressing the phosphorylation of RXR α 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 RXR α . Combined treatment with ACR plus valproic acid, a histone deacetylase (HDAC) inhibitor, acts synergistically to induce apoptosis and G₀-G₁ cell cycle arrest in HCC cells by inhibiting phosphorylation of RXR α , ERK, Akt, and GSK-3 β proteins (13).

In addition to dephosphorylation of RXR α , 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

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RAR β 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 RXR α phosphorylation is profoundly involved in liver carcinogenesis and thus may be a critical target for HCC chemoprevention. Clinical trials reveal that ACR, which inhibits RXR α phosphorylation but induces RAR β 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.

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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; VK₂, vitamin K₂

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Send correspondence to: Masahito Shimizu, Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu, Japan 501-1194, Tel: 81-58-230-6308, Fax: 81-58-230-6310, E-mail: shimim-gif@umin.ac.jp



Molecular and Cellular Pharmacology

Rho-kinase inhibitor upregulates migration by altering focal adhesion formation via the Akt pathway in colon cancer cells

Seiji Adachi ^{a,b,*}, Ichiro Yasuda ^a, Masanori Nakashima ^a, Takahiro Yamauchi ^a, Takashi Yoshioka ^c, Yukio Okano ^c, Hisataka Moriwaki ^a, Osamu Kozawa ^b

^a Department of Gastroenterology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

^b Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

^c Department of Molecular Pathobiochemistry, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan

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ABSTRACT

Although Rho-kinase is reportedly implicated in carcinogenesis and the progression of human cancers, its precise mechanism has not been fully elucidated. We recently reported that Rho-kinase negatively regulates epidermal growth factor (EGF)-stimulated cancer progression in SW480 colon cancer cells. In the present study, we investigated the effect of Rho-kinase on the migration of SW480 colon cancer cells and the mechanism underlying the involvement of Rho-kinase. Interestingly, (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2HCl (Y27632), a specific inhibitor of Rho-kinase, dose-dependently enhanced cell migration. SW480 cells spontaneously release vascular endothelial growth factor (VEGF), however, Y27632 had little effect on its release. While Rho-kinase, which is generally phosphorylated in unstimulated cells, was clearly suppressed by Y27632, exogenous VEGF did not affect its phosphorylation. Immunofluorescence microscopy revealed that Y27632 caused a dramatic change in the localization of focal adhesion components, vinculin, phosphorylated caveolin-1 and tyrosine-phosphorylated proteins in SW480 cells. Furthermore, Akt inhibitor restored the loss of vinculin-stained focal adhesion formation induced by Y27632. We also observed similar effects for Y27632 on the migration and localization of focal adhesion components such as vinculin in another colon cancer cell line, HT29. Taken together, these results strongly suggest that Rho-kinase negatively regulates the migration of colon cancer cells by altering focal adhesion formation via the Akt pathway.

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1. Introduction

Cell invasion into adjacent tissues is a major prognostic factor for advanced colon cancer patients. Abnormal cell migration leads to pathological states such as invasion and metastasis of cancer. The multistep process of cell migration includes the extension of lamellipodia, cell surface protrusions comprised of actin filaments, which are anchored to the underlying substratum by small integrin-dependent focal adhesions (Brew et al., 2009). The cell cytoskeleton is composed of actin filaments and a network of microtubules, and controls cell motility (Ridley et al., 2003). The polymerization of actin pushes against the plasma membrane and provides the force for forward movement. Actin stress fibers generate contractile forces by pulling against focal adhesions to induce retraction of the rear cell membrane, suggesting that stress fibers are important for cell migration (Burrige, 1981). Cytoskeletal proteins such as vinculin, actinin, and several non-receptor protein tyrosine kinases, including

members of the Src family and focal adhesion kinase (FAK), are involved in the organization of focal adhesion complexes (Burrige and Chrzanowska-Wodnicka, 1996; Humphries et al., 2007).

GTPases of the Ras and Rho superfamily act as molecular switches to control a wide range of essential biological pathways in all eukaryotic cells (Bar-Sagi and Hall, 2000). Once they are active, Ras and Rho GTPases interact with target proteins to promote a cellular response (Bar-Sagi and Hall, 2000). Among the molecules in this superfamily, Rho regulates the formation of contractile actin–myosin filaments, which form stress fibers, and maintains focal adhesions at the rear of the cell (Salhia et al., 2005). RhoA reportedly induces the formation of stress fibers and mature focal adhesions through activation of its downstream effectors such as Rho-kinase (Matsui et al., 1996; Pellegrin and Mellor, 2007). Rho GTPases also contribute to the regulation of cell cycle progression, cell motility, and invasive phenotypes (Sahai and Marshall, 2002). Although the overexpression of Rho has been linked to progression of human cancers (Fritz et al., 1999), an opposing effect of Rho in the process of cell migration has also been reported. Dihydromotuporamine C (dhMotC), a macrocyclic alkaloid that inhibits tumor cell invasion, induces the formation of new stress fibers and large focal adhesion complexes that are dispersed around the entire cell periphery. The compound also has

* Corresponding author. Department of Pharmacology, Gifu University Graduate School of Medicine, Gifu 501-1194, Japan. Tel.: +81 58 230 6217; fax: +81 58 230 6218.

E-mail address: seijiadachi0123@gmail.com (S. Adachi).

the ability to activate Rho, and this ability appears to be an important determinant of the anti-invasive activity of dhMotC (McHardy et al., 2004). Therefore, the roles of the Rho GTPases and their downstream effectors in cancer progression and invasion remain controversial.

Rho-kinase is one of numerous targets of Rho (Riento and Ridley, 2003). Accumulating evidence indicates that the Rho/Rho-kinase pathway plays an important role in various cellular functions, such as vascular smooth muscle cell contraction, cell migration and cell proliferation (Sahai and Marshall, 2002). However, the exact role that Rho-kinase plays in cancer cells remains to be elucidated. We recently reported that Rho-kinase negatively regulates epidermal growth factor (EGF)-stimulated cancer progression in SW480 colon cancer cells (Nakashima et al., 2010). In the present study, we investigated the role of Rho-kinase in colon cancer cell migration.

2. Materials and methods

2.1. Materials

(R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide, 2HCl (Y27632), and Akt inhibitor were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Recombinant human VEGF was purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-phospho-specific myosin phosphatase targeting subunit-1 (MYPT-1) (Thr850) antibodies were purchased from Upstate (Lake Placid, NY). Anti-MYPT-1 antibodies and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-specific Akt and Akt were purchased from Cell Signaling, Inc. (Beverly, MA). Antibodies against phospho-caveolin-1 and phosphotyrosine were purchased from BD Transduction Laboratories (San Jose, CA). The ECL Western blot detection system was purchased from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. Y27632 was dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for the Western blot analysis.

2.2. Cell culture

Unless indicated otherwise, SW480 and HT29 human colon cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, San Diego, CA), containing 10% fetal calf serum. Before the experiments, they were incubated in serum free medium for an additional 24 h as described previously (Adachi et al., 2008).

2.3. Measurement of VEGF

The SW480 culture medium was changed to fresh media without serum, and cells were incubated for 0, 12, 24 and 48 h. The respective media were then collected and the VEGF concentration was measured using a human VEGF enzyme-linked immune-sorbent assay (ELISA) kit purchased from R&D Systems, Inc.

2.4. Cell migration assay

Cell migration was assessed using a Boyden chamber (8 mmol/L pores, Transwell®; Corning Costar Corp., Cambridge, MA). The cells (5×10^4 per well) were seeded in the upper chamber, and DMEM containing 10% fetal calf serum and the indicated compounds were added to the bottom chamber. After 48 h incubation at 37 °C, the cells on the upper surface of the membrane were mechanically removed, and the cells that had migrated to the lower surface of the membrane were fixed and stained with hematoxylin. The average number of migrated cells from 5 randomly chosen fields on the lower surface of the membrane was counted. Each experiment was performed in triplicate.

2.5. Western blot analysis

Western blot analyses were performed as described previously (Adachi et al., 2007). In brief, the cells were treated with various concentrations of Y27632 (0–3 μ M) for 60 min and protein extracts were examined by a Western blot analysis. The protein (10 μ g) was fractionated and transferred onto an Immune-Blot PVDF Membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% fat-free dry milk in phosphate buffered saline containing 0.1% Tween-20 for 30 min before incubation with the indicated primary antibodies. Peroxidase-labeled antibodies were used as secondary antibodies. The peroxidase activity on the membrane was visualized on X-ray film by means of the ECL Western blot detection system.

2.6. Immunofluorescence microscopy studies

Immunofluorescence microscopy studies were performed as described previously (Adachi et al., 2009a,b). The cells grown on coverslip-bottom dishes were incubated with or without Y27632 (3 μ M) for 60 min at 37 °C. The cells were then fixed with 4% paraformaldehyde for 10 min on ice and exposed to 0.1% Triton X-100 for 10 min to permeabilize the cell membrane. They were then exposed to the indicated primary antibodies, followed by exposure to Alexa Fluor conjugated secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) (Wako, Tokyo, Japan) for 60 min. Finally, the cells were examined by fluorescence microscopy using a BIOREVO system (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer's protocol.

2.7. Densitometric analysis

The densitometric analysis was performed using a scanner and an image analysis software package (Image J ver. 1.32). The background-subtracted signal intensity of each protein signal was normalized to the respective control signal. All data were obtained from at least three independent experiments.

2.8. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between the indicated pairs, and $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of Rho-kinase inhibitor on the migration of SW480 and HT29 colon cancer cells

We first investigated the effect of Y27632, a specific inhibitor of Rho-kinase (Shimokawa and Rashid, 2007), on cell migration in SW480 and HT29 cells. As shown in Fig. 1, we examined cell motility using a Boyden chamber and found that 3 μ M of Y27632 significantly stimulated the migration of SW480 cells (Fig. 1A). Y27632 also dose-dependently enhanced the migration of HT29 cells (Fig. 1B), suggesting a negative role for Rho-kinase in colon cancer cell migration. Of interest, we recently reported the inhibition of Rho-kinase to stimulate colon cancer cell proliferation (Nakashima et al., 2010). These results led us to further investigate the mechanism underlying the involvement of Rho-kinase in colon cancer cell migration.

3.2. Effect of Rho-kinase inhibitor on VEGF release in SW480 colon cancer cells

VEGF has been well documented to be the most potent inducer of angiogenesis, while also promoting several events required for the formation of new blood vessels, such as endothelial cell proliferation,

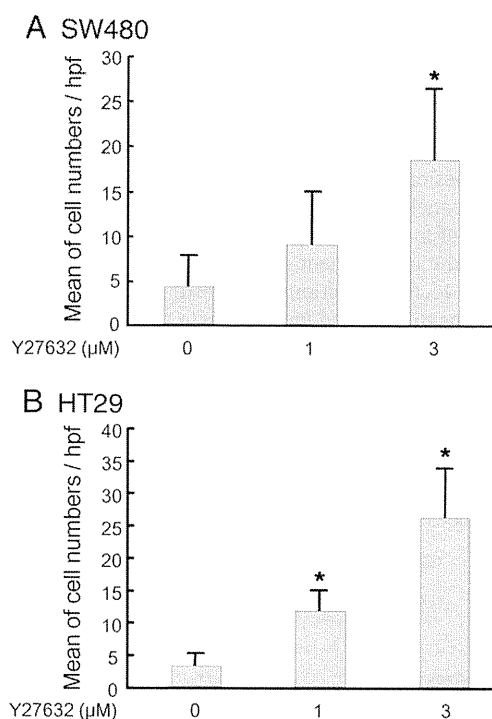


Fig. 1. The effects of Y27632 on the migration of SW480 and HT29 colon cancer cells. The SW480 (A) and HT29 (B) cells (5×10^4 per well) were seeded in the upper Boyden chamber in DMEM containing 10% fetal calf serum, and after 24 h incubation, various concentrations of Y27632 were added to the bottom chamber and cells were incubated for an additional 48 h at 37 °C. Next, the remaining cells on the upper surface of the membrane were mechanically removed, and the cells that had migrated to the lower surface of the membrane were fixed and stained with hematoxylin. The average number of migrated cells from 5 randomly chosen fields on the lower surface of the membrane was counted. Each value represents the mean \pm S.E.M. of triplicate independent determinations. * indicates a significant increase ($P < 0.05$) compared with the control (lane 1, respectively).

migration and vascular permeability, all of which can lead to metastasis (Pourgholami and Morris, 2008). Therefore, we next measured the VEGF concentration in the medium of SW480 cells to determine whether these cells are able to produce VEGF. After incubation of the cells in the medium containing 10% fetal calf serum, they were cultured in fresh medium without serum for the indicated periods. As a result, the VEGF concentration was gradually increased (Fig. 2A, open circle), thus suggesting that SW480 cells can produce VEGF.

Since we found that Y27632 caused the migration of colon cancer cells (as described above; Fig. 1), we next investigated the effect of Y27632 on VEGF release from SW480 cells. However, Y27632 did not affect its release (Fig. 2A, closed circle). This suggests that the increase in migration by the cells incubated with Y27632 is not due to an increase in VEGF release from the SW480 cells.

3.3. Effect of exogenous VEGF on Rho-kinase activity in SW480 colon cancer cells

We next examined the effect of exogenous VEGF on the levels of phosphorylated MYPT-1, which is a component of myosin phosphatase and well known as a downstream substrate of Rho-kinase (Riento and Ridley, 2003). We observed that MYPT-1 was phosphorylated even in untreated SW480 cells (Fig. 2B, lane 1), which is consistent with our previous study (Nakashima et al., 2010). However, when the cells were exposed to exogenous VEGF, the phosphorylated levels of MYPT-1 was not affected (Fig. 2B, lane 2 compared to lane 1). We also examined the effect of various concentrations of VEGF for different periods of time (up to 180 min) on the phosphorylation of MYPT-1, but did not observe any increase in the phosphorylation level (data

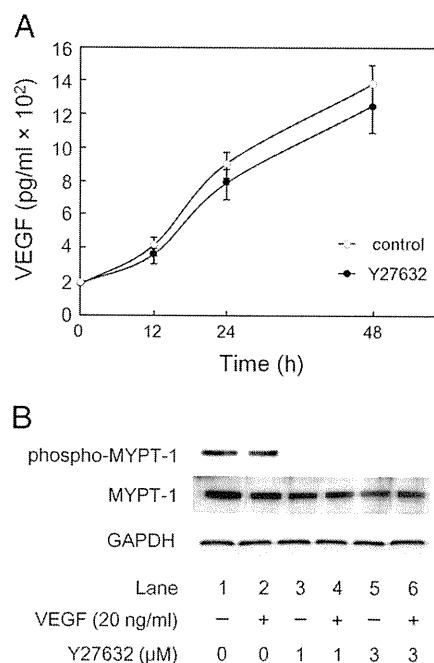


Fig. 2. (A) The effect of Y27632 on VEGF release in SW480 colon cancer cells. After incubation in the medium containing 10% fetal calf serum, the cells were cultured in fresh medium without serum and incubated for the indicated periods. The respective media were then collected, and the VEGF concentration was measured by ELISA (see Materials and Methods). Open circle (○): untreated control cells. Closed circle (●): 3 µM of Y27632-treated cells. Each value represents the mean \pm S.E.M. of triplicate independent determinations. (B) The effects of VEGF and Y27632 on the phosphorylation of MYPT-1 in SW480 colon cancer cells. SW480 cells were pretreated with various concentrations of Y27632 for 60 min, followed by exposure to 20 ng/mL of VEGF or vehicle for 20 min. The extracts of cells were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were then subjected to the Western blot analysis with antibodies against phospho-specific MYPT-1, MYPT-1 and GAPDH.

not shown). However, we verified that Y27632 clearly suppressed the phosphorylation of MYPT-1 at a concentration of 1 µM or greater (Fig. 2B, lanes 3 to 6), while Y27632 did not affect the total protein levels of MYPT-1 (Fig. 2B, lower panel). Based on our findings, it is most likely that Rho-kinase is generally in an activated state in unstimulated SW480 cells, and exogenous VEGF therefore has little effect on the activation of Rho-kinase in these cells.

3.4. Effect of Rho-kinase inhibitor on the localization of focal adhesion components in SW480 colon cancer cells

We next performed an immunofluorescence microscopy study to observe the abundance and localization of several cytoskeletal proteins, such as vinculin, because cell migration involves changes in the cytoskeleton and cell adhesion (Ridley et al., 2003). In untreated SW480 cells, vinculin, which is a characteristic feature of focal adhesion formation (Brew et al., 2009), was strongly stained on focal adhesions around the cell periphery (Fig. 3, panel 1), where the stress fiber terminates (Brew et al., 2009). When SW480 cells were pretreated with Y27632, there was a marked loss in the size and number of focal adhesions (as indicated by vinculin staining) around the cell periphery (Fig. 3, panel 2 compared to panel 1). Moreover, the expression and localization of phosphorylated caveolin-1, another component of the focal adhesion complex (Joshi et al., 2008), were similar to vinculin (Fig. 3, panel 3), and incubation with Y27632 also caused the loss of the localization of phosphorylated caveolin-1 (Fig. 3, panel 4).

Several non-receptor protein kinases, including members of the Src family and FAK, are involved in the organization of molecular adhesion complexes (Burridge and Chrzanowska-Wodnicka, 1996;

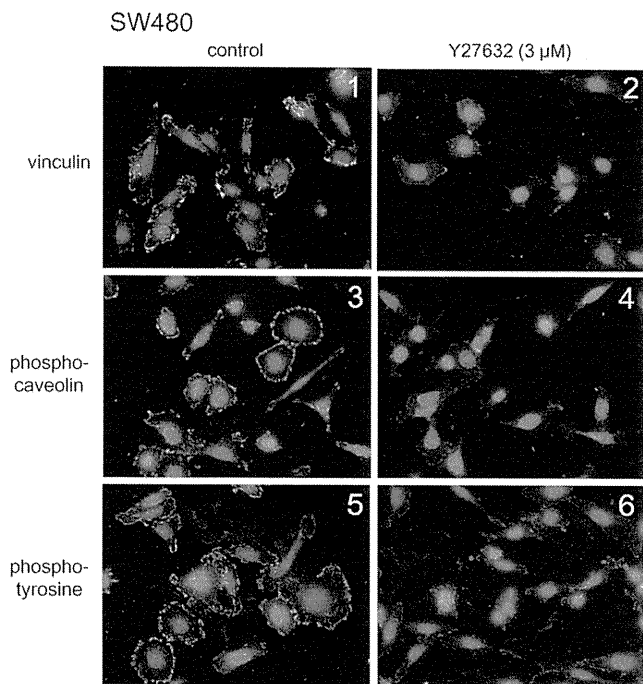


Fig. 3. The effect of Y27632 on the formation of focal adhesion complexes in SW480 colon cancer cells. SW480 cells grown on coverslip-bottom dishes were incubated with (panels 2, 4 and 6) or without Y27632 (panels 1, 3 and 5) for 60 min at 37 °C. After fixation, the cells were exposed to anti-vinculin antibodies (panels 1 and 2), anti-phospho-caveolin-1 antibodies (panels 3 and 4) and anti-phosphotyrosine antibodies (panels 5 and 6), followed by exposure to Alexa Fluor 488® conjugated secondary antibodies and DAPI for 60 min. The cells were then examined by fluorescence microscopy.

Humphries et al., 2007) and they regulate the signaling events that occur at focal adhesions (Wozniak et al., 2004). To examine the effect of Y27632 on the localization of tyrosine-phosphorylated proteins at focal adhesions, we used antibodies against pan-phosphotyrosine. In untreated SW480 cells, anti-phosphotyrosine staining was concentrated mostly at the cell edges, similar to that observed for vinculin or phosphorylated caveolin-1. Y27632 also caused the loss of localization of these tyrosine-phosphorylated proteins (Fig. 3, panels 5 and 6). These results suggest that Y27632 causes a dramatic change in the localization of focal adhesion components such as vinculin, phosphorylated caveolin-1 and tyrosine-phosphorylated proteins, thereby supporting our findings that Y27632 induced the migration of colon cancer cells as shown in Fig. 1.

3.5. Effect of Rho-kinase inhibitor on the Akt pathway in SW480 colon cancer cells

We next investigated the effect of Y27632 on the Akt pathway in SW480 cells. Y27632 markedly induced the phosphorylation of Akt in a time-dependent manner (Fig. 4A). The effect of Y27632 on the phosphorylation of Akt was observed within 1 h and reached its maximum at 3 h, and decreased thereafter. We also observed a similar effect in the cells treated with another Rho-kinase inhibitor, fasudil (Shimokawa and Rashid, 2007) (data not shown).

GSK-3 β is a critical downstream element of the PI3K/Akt cell survival pathway, and its activity can be inhibited by Akt-mediated phosphorylation (Cross et al., 1995). Therefore, we next examined the effect of Y27632 on the level of phosphorylated GSK-3 β . Y27632 caused the phosphorylation of GSK-3 β within 30 min, which was sustained for 24 h, and decreased thereafter (Fig. 4A). We further confirmed that Y27632-induced phosphorylation of Akt was significantly suppressed when the cells were treated with Akt inhibitor.

3.6. The involvement of the Akt pathway in the Y27632-induced alteration of focal adhesion formation in SW480 and HT29 colon cancer cells

As described earlier, we showed strong staining with vinculin in the focal adhesions around the cell periphery in untreated SW480 cells (Fig. 3, panel 1), and that Y27632 caused a decrease in the size and number of focal adhesions (Fig. 3, panel 2). Therefore, we next investigated the involvement of Akt in focal adhesion formation. As shown in Fig. 4C, the inhibition of Akt restored the size and number of focal adhesions that stained for vinculin in the SW480 cells incubated

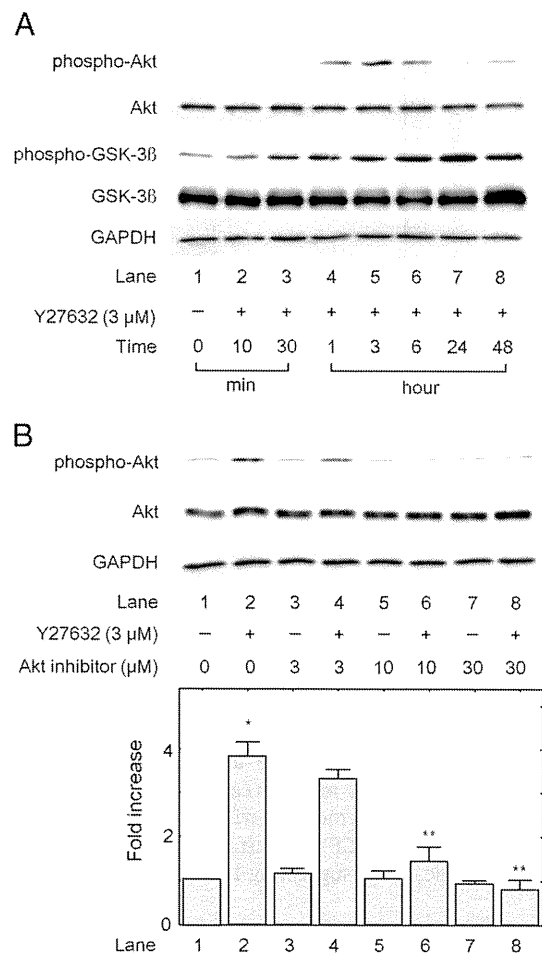


Fig. 4. The involvement of Akt in SW480 cell migration. (A) The effect of Y27632 on the phosphorylation of Akt and GSK-3 β in SW480 colon cancer cells. Cells were pretreated with 3 μ M of Y27632 for the indicated periods, and the extracts of cells were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Western blot analysis with antibodies against phospho-specific Akt, Akt, phospho-specific GSK-3 β , GSK-3 β and GAPDH as a loading control. (B) The effect of the Akt inhibitor on Y27632-induced phosphorylation of Akt in SW480 colon cancer cells. The cells were pretreated with the indicated concentrations of Akt inhibitor for 60 min, and then were exposed to 3 μ M of Y27632 for 60 min. The extracts of cells were then subjected to SDS-PAGE and a Western blot analysis with antibodies against phospho-specific Akt, Akt and GAPDH as a loading control. The lower bar graph shows the quantification data for the relative levels of phospho-Akt, after normalization with respect to total Akt, as determined by densitometry. Each value represents the mean \pm S.E.M. of triplicate independent determinations. * indicates a significant increase ($P < 0.05$, compared with lane 1) and ** indicates a significant decrease ($P < 0.05$, compared with lane 2). (C and D) The effect of the Akt inhibitor on Y27632-induced alteration of the localization of focal adhesion molecule, vinculin, in SW480 (C) and HT29 (D) colon cancer cells. The cells grown on coverslip-bottom dishes were pretreated with or without the Akt inhibitor (10 μ M) for 60 min and then incubated with or without Y27632 (3 μ M) for 60 min at 37 °C. After fixation, the cells were then exposed to anti-vinculin antibodies, followed by exposure to Alexa Fluor 488® conjugated secondary antibodies and DAPI for 60 min. The cells were then examined by fluorescence microscopy.