Molecular mechanism for growth suppression of human hepatocellular carcinoma cells by acyclic retinoid

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We have reported previously that acyclic retinoid, a synthetic retinoid X receptor α (RXRα)-ligand, suppresses the development of hepatocellular carcinoma (HCC) in patients with chronic liver disease. On the other hand, HCCs become refractory to physiological concentrations of the natural RXRα-ligand, 9-cis retinoic acid (9cRA), due to extracellular signal-regulated kinase (Erk) 1/2-mediated phosphorylation and inactivation of RXRα. Here, we show that acyclic retinoid restores the function of RXRα in human HCC-derived HuH7 cells by inactivating the Ras-Erk 1/2 signaling system, thereby dephosphorylating RXRα. In contrast, 9cRA failed to suppress phosphoErk 1/2 levels and subsequent RXRα phosphorylation. Although 9cRA also suppressed Ras activity, it simultaneously down-regulated mitogen-activated protein kinase phosphatase-1, an enzyme that inactivates Erk, thereby leaving the phosphorylation status of Erk unchanged. A combination of 9cRA (a potent ligand) and acyclic retinoid (a weak ligand preventing phosphorylation) resulted in a marked cooperation in transactivation via the RXR-response element and in inhibiting the proliferation of HuH7 cells. These events provide a novel molecular basis for the antitumor activity of acyclic retinoid against HCC.

Introduction

Retinoids are prime candidates for cancer chemoprevention, as they have profound effects on cell growth, apoptosis and differentiation (1). In fact, experimental studies have revealed potent roles of retinoids in reversing the carcinogenesis process and suppressing malignant transformation (2). Loss of retinoid activity or responsiveness is closely linked to carcinogenesis in several organs including hepatocellular carcinoma (HCC) (3). We documented previously a local depletion of retinoid in human HCC tissues due to its rapid conversion to an inactive metabolite (3) and further showed in experimental and clinical studies that supplementation with a synthetic retinoid analogue, acyclic retinoid, suppresses the development of HCC (4,5). However, the molecular mechanism(s) underlying this effect has been unclear.

Retinoids exert their biological activities primarily through retinoic acid receptor (RAR) and retinoid X receptor (RXR), two members of the nuclear receptor superfamily that act as ligand-dependent transcriptional regulators (6). RXR interacts with both all-trans retinoic acid (atRA) and 9-cis RA (9cRA), whereas RXX binds only to 9cRA. Acyclic retinoid is a ligand of both RAR and RXR (7). Both RAR and RXR consist of three subtypes, α, β and γ, characterized by a modular domain structure. RXR forms a homodimer as well as heterodimers with RAR and other nuclear receptors, including vitamin D receptor (VDR), thyroid hormone receptor and peroxisome proliferator-activated receptor (PPAR), and thereby participates in the regulation of respective downstream genes. Among three subtypes of RXR, RXRα is highly expressed in human HCC and HCC-derived HuH7 cells (8). These dimers bind to their respective response elements and subsequently activate or inhibit the expression of target genes. Transcriptional activity of retinoid receptors is regulated by several factors that modulate the composition of their receptor complexes (7). In the absence of RA, unliganded RAR/RXR heterodimer binds to co-repressor complexes that provide a link between the heterodimer and histone deacetylases, resulting in chromatin condensation and gene silencing by removing acetyl groups from nucleosomal histones. Ligand binding to the retinoid receptors induces their conformational change, which allows the interaction between RAR/RXR and co-activators. Co-activators form multiprotein complexes that have histone acetyltransferase activity to acetylate histone N-terminal tails. Acetylation of histones leads to nucleosomal repulsion and chromatin decondensation, which is thought to be indispensable for the transcriptional activation by retinoid receptor.

We have found that malfunction of RXRα due to post-translational modification by phosphorylation is associated with carcinogenesis of HCC (8,9) in addition to depletion of hepatic retinoid (3). Phosphorylation is reported to enhance or suppress the function of nuclear receptors in a context-dependent manner. Solomon et al. (10) have shown that in ras-transfected keratinocytes phosphorylation of RXRα at serine 260 by activated Ras-extracellular signal-regulated kinase (Erk) 1/2 [also known as mitogen-activated protein (MAP) kinase] pathway results in attenuated transactivation by VDR/RXRα heterodimer and consequently in resistance to the growth inhibitory effect of 1,25-dihydroxyvitamin D3 and RXR-specific agonist. Similarly, we have also demonstrated that phosphorylation of RXRα at serine 260 by Erk 1/2 impairs RXRα function and its normal degradation through the ubiquitin–proteasome pathway in both human HCC tissues and HuH7 human HCC cells (9,11). This leads

Abbreviations: 4HPR, 4-hydroxyphenyl retinamide; 9cRA, 9-cis RA, retinoic acid; atRA, all-transRA; CRBP, cellular retinol-binding protein; EGF, epidermal growth factor; Erk, extracellular signal-regulated kinase; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; MAP kinase, mitogen-activated protein kinase; MKP-1, MAP kinase phosphatase-1; RAR, retinoic acid receptor; RXR, retinoid X receptor; RXRE, RXR-response element; TGF-α, transforming growth factor-α; VDR, vitamin D receptor.
to accumulation of non-functional phosphoRXRα in the cancer cells, which may interfere with the function of remaining normal RXRα in a dominant-negative manner, thereby rendering the cancer cells much less sensitive to 9cRA. Hence, HCC cells evade 9cRA-induced apoptosis (12). In contrast, HCC cells are sensitive to acyclic retinoid and undergo apoptosis (12). Because over-expression of phosphorylation-site mutated unphosphorylated RXRα restored its activity and suppressed tumor cell growth (9), we have examined whether acyclic retinoid can suppress RXRα phosphorylation and thereby restore its function in HCC cells.

We have pursued this possibility using HuH7 cells, which reveal that acyclic retinoid dephosphorylates RXRα by inactivating the Ras-Erk system in HCC cells, potentiating the responses of RXRα to physiological concentration of 9cRA. Our findings identify these events as a key molecular mechanism underlying the anti-tumor activity of acyclic retinoid.

Materials and methods

Materials

Acyclic retinoid (NIK333) was supplied from Nikken Chemicals Co. (Tokyo, Japan) (3). 9cRA, lactoalumin hydrolysat and monoclonal antibody against phosphoserine (PSR-45) were purchased from Sigma Chemical (St Louis, MO). Polyclonal anti-RXRα (DN197) and polyclonal anti-MAP kinase phosphatase-1 (MKP-1) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Erk 1/2 antibodies and polyclonal anti-phosphoErk 1/2 antibodies were from Cell Signaling Technology (Beverly, MA). Monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon International (Temecula, CA). RPMI 1640 media and fetal calf serum (FCS) were from Invitrogen (Carlsbad, CA).

Cell culture and treatment

HuH7 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) (13) and were plated on 100 mm-plastic dishes (8 x 10⁵ cells/dish) or 96-well plates (1.75 x 10³ cells/well) at the cell density of 30–40% confluency and were treated for 24 h in the same medium with the indicated concentrations of retinoids dissolved in ethanol at a final concentration of 0.05%. Cell numbers were counted using Trypan Blue dye exclusion method.

Preparation of cell extracts and western blot analysis

Preparation of cell extracts and western blot analyses of total Erk 1/2, phosphoErk 1/2, MKP-1 and GAPDH were performed as described previously (9).

Fig. 1. Effects of retinoids on the levels of phosphoserine RXRα (A) and phosphoErk 1/2 (B) in HuH7 cells. HuH7 cells were treated with vehicle (panel A and B, lanes and column 1), 1 μM acyclic retinoid (lanes and column 2), 1 μM 9cRA (lanes and column 3) or their combination (lanes and column 4) for 24 h. (A) RXRα was affinity-purified from the cell extract using anti-RXRα antibody-immobilized Sepharose beads, and then subjected to western blot analyses employing anti-phosphoserine antibody (9) as a control. Proteins precipitated with non-immunized rabbit antibody-linked beads were analyzed. Protein concentrations in the samples were determined by Bio-Rad protein assay kit (Hercules, CA). The signals were detected with an Amersham Bioscience ECL system (Piscataway, NJ). Densitometric analysis was performed using the NIH image version 1.61 software.

Results

Acyclic retinoid reduced phosphoserine RXRα levels and Erk activity

In our previous study, we validated human HCC-derived HuH7 cells as a faithful representative of clinical HCC tissues in terms of MAP kinase-mediated phosphorylation of RXRα (9). Therefore, we have used HuH7 cells in the present study to examine if acyclic retinoid might prevent the phosphorylation of RXRα and restore its function. Because malfunction of RXRα is due to phosphorylation at a serine residue (9), we first examined if acyclic retinoid might dephosphorylate a phosphoseren RXRα. As depicted in Figure 1A, levels of phosphoserine RXRα (lane 1) band was reduced markedly following treatment with acyclic retinoid alone (lane 2) and
with the combination of acyclic retinoid plus 9cRA (lane 4), but not with 9cRA alone (lane 3).

We have shown previously that Erk 1/2, constitutively expressed in the HCC cells (11), plays a crucial role in RXRa phosphorylation (9). We thus examined if acyclic retinoid might suppress Erk activity. As depicted in Figure 1B, the amount of phosphoErk 1/2, active forms of Erk, was also reduced significantly \((P < 0.05)\) following treatment with acyclic retinoid alone (lanes and column 2) and with the combination of acyclic retinoid plus 9cRA (lanes and column 4), but not with 9cRA alone (lanes and column 3), as has been reported previously (11). The amount of total Erk 1/2 was not affected by either treatment (Figure 1B). These results suggest that HuH7 cells are refractory to 9cRA, but sensitive to acyclic retinoid, in regulating the activation of Erk 1/2 and subsequent RXRa phosphorylation.

**Differential regulation of Ras and MKP-1 levels between acyclic retinoid and 9cRA**

Our previous work suggested that the down-regulation of transforming growth factor (TGF-\(\alpha\)) interrupted autocrine growth signals via the epidermal growth factor (EGF) receptor, the receptor for TGF-\(\alpha\), providing a potential mechanism for apoptosis induction by acyclic retinoid in HuH7 cells (15). Therefore, we examined if acyclic retinoid might downregulate the activity of Ras, a small GTP-binding protein, which mediates activation of the MAP kinase pathway following stimulation by TGF-\(\alpha\) (16). As shown in Figure 2A, Raf-1-bound Ras activities were inhibited by 70% in acyclic retinoid-treated HuH7 cells (lane and column 2). Similar inhibition was also observed following 9cRA-treatment (Figure 2A, lane and column 3). To provide an explanation for the discrepancy between 9cRA’s effects on Ras activity (suppressed; Figure 2A, lane and column 3) and on phosphoErk levels (unchanged; Figure 1B, lanes and column 3), we analyzed the effect of 9cRA on the levels of MKP-1, a phosphatase that inactivates Erk 1/2. As depicted in Figure 2B, 9cRA suppressed MKP-1 levels by 40\% (lanes and column 3), whereas acyclic retinoid did not reduce the levels of MKP-1 either in the absence (lanes and column 2) or in the presence of 9cRA (lanes and column 4). These results suggest that because 9cRA suppresses simultaneously both activating protein (Ras) and inactivating enzyme (MKP-1), 9cRA leaves the levels of phosphoErk 1/2 unaffected. In contrast, as acyclic retinoid downregulates Ras activity without accompanying a decrease in MKP-1 levels, it suppresses phosphoErk 1/2 levels and prevents generation of phosphoRXRa.

**Acyclic retinoid potentiates 9cRA-induced transactivation via RXRE**

We have shown previously that compared with phosphorylated RXRa, an unphosphorylated form of RXRa exerts much higher transactivation activity in response to 9cRA (9). Acyclic retinoid at \(\geq 5 \mu M\) can serve as a ligand for RXRa, but has much less potent transactivation activity than that of 9cRA (7,17,18). Together with the results obtained in the present study, the data suggest that 9cRA cannot prevent phosphorylation of RXRa, but is a potent ligand when RXRa is kept unphosphorylated, whereas acyclic retinoid can prevent

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**Fig. 2.** Effects of retinoids on the levels of activated Ras (A) and MKP-1 (B) in HuH7 cells. HuH7 cells were treated with vehicle (panels A and B, lanes and columns 1), 1 \(\mu M\) acyclic retinoid (lanes and columns 2), 1 \(\mu M\) 9cRA (lanes and columns 3) or their combination (panel B, lanes and column 4) for 24 h. (A) Levels of Ras bound to Raf-1. Ras was precipitated in the cell extract using Raf-1/Ras-binding domain-immobilized agarose and then subjected to western blot analysis using anti-Ras antibody. (B) Western blot analyses of MKP-1 and GAPDH were performed with the respective specific antibodies using the same extract as in panel A. (A) Relative intensity of the band was determined by densitometry (columns 1–3). Values are the mean ± SD \((n = 5)\). Double asterisks represent significant difference \((P < 0.01)\) compared to column 1. (B) Values represent the amounts of MKP-1 divided by those of GAPDH (the mean ± SD, \(n = 5\)). An asterisk represents significant difference \((P < 0.05)\) compared with columns 1, 2 and 4.
the phosphorylation, but is a weak ligand. This led us to explore whether a combination of 9cRA (a potent ligand) and acyclic retinoid (a weak ligand preventing phosphorylation) might exert a cooperativity on transactivation via RXRE. This suggestion was borne out in the findings illustrated in Figure 3.

HuH7 cells, which had been transfected with an RXRE-containing gene promoter, were treated with incremental concentrations of 9cRA or acyclic retinoid alone, or with a combination of incremental concentrations of 9cRA and a fixed concentration (1 μM) of acyclic retinoid, and then transactivation activity was measured. 9cRA induced transactivation in a dose-dependent manner at ≥100 nM (Figure 3, curve 2). These activities were further enhanced ~20-fold by simultaneous addition of acyclic retinoid (Figure 3, curve 3), which alone had no transactivating activity (curve 1). In the previous report we found that acyclic retinoid alone induced RXRE-mediated transactivation activity (17). Although we cannot explain the reason for this discrepancy, we speculate that the differences in the experimental condition (techniques employed for transfection, culture media conditions and cell densities) would be responsible for it.

This current result suggests that there might be a 20-fold molar excess of non-functioning phosphoRXRα compared with normal RXRα in the cancer cells. Importantly, even 9cRA at physiological concentrations as low as 1–10 nM (6,19), which alone was not transactivating, became active in conjunction with 1 μM acyclic retinoid, a pharmacological concentration in patients’ plasma (4). These results suggest that prevention of RXRα phosphorylation by acyclic retinoid might be linked to the restoration of its function and transactivation activity in response to physiological concentration of 9cRA in vivo.

Acyclic retinoid and 9cRA cooperatively suppress proliferation of HuH7 cells

We have suggested previously that malfunction of RXRα due to phosphorylation appears to be associated with aberrant proliferation of HuH7 cells, and that introduction of an unphosphorylated form of RXRα suppresses cell growth (9). Therefore, we examined if the combination of acyclic retinoid plus 9cRA might cooperatively suppress the growth of HuH7 cells (Figure 4). To see clearer differences in the growth suppression between single treatment with each of two retinoids and their combination, we used the culture medium containing FCS, which was known to regress the effect of acyclic retinoid (13). Acyclic retinoid alone at concentrations ≤1 μM minimally suppressed cell growth (<3%; Figure 4, curve 1) as we have reported previously (12). 9cRA was not effective until 100 nM, and slightly suppressed the growth at 1 μM, culminating in a 23% inhibition (Figure 4, curve 2). In contrast, in conjunction with 1 μM acyclic retinoid, 9cRA suppressed cell growth from 0.1 nM and resulted in 25% inhibition at 10 nM (Figure 4, curve 4). Growth suppression by 9cRA was also restored when HuH7 cells were transfected with unphosphorylated mimic of RXRα (S260A mutant;...
Figure 4, curve 3). These results suggest that prevention of phosphorylation of RXRα and thus restoration of its function by acyclic retinoid enhance the susceptibility of cancer cells to 9cRA, enabling growth suppression at physiological concentrations. This mechanism may provide a potential explanation for inhibition of the development of HCC by acyclic retinoid in vivo, although other possibilities remain.

**Discussion**

We here demonstrate a novel action of a synthetic retinoid, acyclic retinoid, to prevent phosphorylation of RXRα (Figure 1A) by inhibiting the activities of Ras-Raf-Erk 1/2 system (Figures 1B and 2A), and thereby to restore the function of RXRα in HCC cells (Figure 3) in addition to its previously established function as an RXR-ligand (7). 9cRA, a natural RXRα-ligand, failed to suppress the RXRα phosphorylation (Figure 1A), probably because 9cRA inhibited both activated Ras (activating protein of Erk) and MKP-1 (inactivating enzyme of Erk) (Figure 2) at the same time, thereby leaving the Erk 1/2 activity unchanged (Figure 1B). Growth suppression by 9cRA was restored when HuH7 cells were either co-treated with acyclic retinoid or transfected with unphosphorylated mimic of RXRα (S260A RXRα; Figure 4).

Thus, the dephosphorylating ability of acyclic retinoid may play a key role in its growth inhibitory effect on HCC (Figure 5). The concentration of the respective retinoid used in the present study (1 μM for acyclic retinoid and 10 nM for 9cRA) is within the physiological ranges (4,6,19), implying that acyclic retinoid and 9cRA may work cooperatively in vivo in the liver.

Retinoid receptors have anticancer properties (1,6) and loss of the receptors is largely involved in the carcinogenic process of various types of cancers (20–25). In particular, recent studies indicate that RXRα is required for apoptosis-induction and/or growth suppression of cancer cells via an autonomous RXR pathway (26,27) or by heterodimerization with other nuclear receptors (10,28). Thus, restoration of impaired RXRα activity may provide a key strategy to render HCC cells sensitive to RA and thus prevent cancer development (29). Solomon et al. originally suggested a similar strategy to use an Erk inhibitor (e.g. PD98059) to prevent phosphorylation of RXRα and restore its function as well as consequent vitamin D signaling via VDR/RXRα heterodimer, thereby reversing the resistance of cancer cells to 1,25-dihydroxyvitamin D3 (30). The novel activities of acyclic retinoid as an Erk inhibitor may provide a clue in uncovering the mechanism of its specific antitumor activity against HCC. Although the ligand activity of acyclic retinoid is much weaker than that of 9cRA (Figure 3), the retinoid might work cooperatively with endogenous 9cRA in vivo and thereby suppress the growth of the tumor whose RXRα is phosphorylated. We have found recently that potential downstream genes inhibiting the cancer cell growth include STAT1 (31) and p21 (18).

The detailed molecular mechanism by which acyclic retinoid suppresses RXRα phosphorylation remains to be elucidated. Others have reported a similar effect of atRA in inhibiting Erk 1/2 activation induced by pro-angiogenic agents in the endothelial cells; however, the molecular mechanisms also remain obscure (32). Recently, Sah et al. (33) have reported that an RAR-selective retinoid inhibits EGF-induced Erk 1/2 activation and subsequent cell proliferation in a human

![Fig. 5. Schematic representation of the effect of acyclic retinoid on RXRα phosphorylation in HCC cells and its implication for specific antitumor activity of the retinoid.](image-url)
uterine cervical cell line. In that study the retinoid seemed to act in an RAR-dependent manner because the effect is reversed by co-treatment with an RAR antagonist (33). In contrast, in the present study acyclic retinoid suppressed Erk activities even in HCC cells in which RXRα function is impaired, suggesting the possibility of RXRα-independent mechanism. In fact, our preliminary data suggest that the inhibitory effect of acyclic retinoid on Erk does not require de novo protein synthesis (Matsushima-Nishiwaki et al., unpublished observation), suggesting a dispensability of retinoid receptor-mediated transactivation, which often accompanies autoinduction of the receptors (20,22,25). Thus, acyclic retinoid might exert its antitumor activity independently of nuclear receptors, as has been reported with other synthetic retinoids including 4-hydroxyphenyl retinamide (4HPR) and CD437 (34–40). Our previous study demonstrated that acyclic retinoid downregulates TGF-α expression in HuH7 cells, and that inclusion with TGF-α prevents acyclic retinoid-induced apoptosis in the cells (15). Because TGF-α is a ligand for EGF receptor, acyclic retinoid might directly interfere with the signal transducing sequence of TGF-α-EGF receptor-Ras-Raf-Erk system.

Phosphorylation has recently been shown to modulate the function of many nuclear receptors. The effect of phosphorylation on the function of each receptor is distinct depending upon the type of the receptor and/or kinases involved as well as upon the sites of phosphorylation. Phosphorylation of RXRα at Tyr249 by MAP kinase kinase-4 and by its downstream mediator c-Jun N-terminal kinase (JNK) has been reported, which results in the reduced transactivation via RAR/RXR complex (41). In contrast, others have reported that RXRα phosphorylation by JNK does not affect the transactivating properties of either RXRα homodimer or RXRα/RXRα heterodimer (42). On the other hand, phosphorylation of RAR seems to enhance its transcriptional activities, irrespective of RAR subtypes and types of kinases (43–45). Much like RXR/RAR, controversy persists regarding the phosphorylation of PPAR, depending upon the experimental conditions (46–48). It is intriguing to see if acyclic retinoids affect phosphorylation of other nuclear receptors and their biological activities.

HCC has become one of the most frequent cancers in the world (49). HCC arises in patients with chronic viral hepatitis and cirrhosis at a high rate (3–5% annually) and is a major cause of morbidity and mortality in patients with advanced liver disease. Thus, prevention of HCC by targeting such a high-risk group is of great significance. We have successfully prevented the development of HCC with the use of acyclic retinoid in a clinical trial (4,5). In that study, we proposed a concept of ‘clonal deletion’ therapy, in which the retinoid eradicates occult malignant cell clones under the detection limit of diagnostic images by inducing apoptosis (50). The present study may suggest a specific molecular mechanism by which acyclic retinoid renders HCC cell clones susceptible to the retinoid therapy and thereby removes the invisible cancer cells.

**Acknowledgements**

This study was supported partly by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (12670472 to M.O., 13670579 to S.K. and 10557055 to H.M.), the Nagoeno Memorial Foundation (to M.O.), the Liver Forum in Kyoto (to M.O.), and the NIDDK (RO1DK 37340 to S.L.F.). The authors thank Dr Y.Muto (Sugiyama Jogakuen University) for the critical reading of the manuscript.

**References**

Acyclic retinoid prevents RXRα phosphorylation


Received August 21, 2002; revised April 4, 2003; accepted April 9, 2003

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