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RETINOIDS IN LIVER FIBROSIS AND CANCER

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1. ABSTRACT

Pathobiological functions and metabolism of retinoids (vitamin A and its derivatives) in liver fibrosis and hepatocellular carcinoma (HCC) are discussed in the present review. Retinoic acid (RA, active metabolite) exacerbates liver fibrosis that is not accompanied by hepatic necroinflammation, in which RA acts directly on hepatic stellate cells (HSCs); RA enhances plasminogen activator/plasmin levels and thereby induces proteolytic activation of latent transforming growth factor-β (TGF-β), a strong fibrogenic cytokine, resulting in enhanced collagen production. We have developed a protease inhibitor, camostat mesilate, that suppresses TGF-β ctivation and thereby inhibits the transformation of HSCs, leading to reduced matrix production by the cells. The compound is effective not only in preventing but also in reducing hepatic fibrosis in rats when administered orally.

HCC is refractory to RA due to its local depletion in the tumors and also due to malfunction of its nuclear receptor, retinoid X receptor-α (RXRα) Oral supplementation of a synthetic retinoid named acyclic retinoid led to the disappearance of serum lectin-reactive α-fetoprotein (AFP-L3) and subsequently suppressed posttherapeutic recurrence of HCC in cirrhotic patients. These results suggest eradication of AFP-L3-producing latent malignant clones from the liver by the retinoid. We propose the concept of "clonal deletion" therapy for cancer chemoprevention, a new category of cancer chemotherapy.

2. INTRODUCTION

Vitamin A and its analogs, collectively termed retinoids, have profound effects on cell activities, including

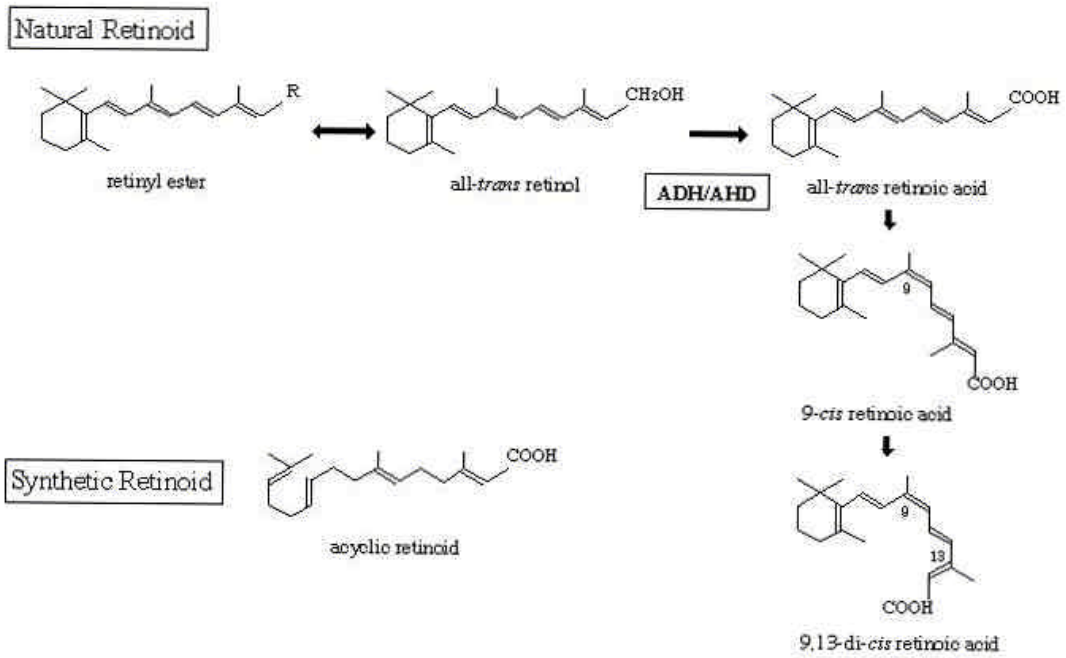

Figure 1. Chemical structures of natural and synthetic retinoids. Retinyl esters (mainly retinyl palmitate, R: fatty acid) stored in the liver are hydrolyzed to retinol that is then transported to target cells after binding to RBP through the circulation. RA is generated from retinol via intermediate retinal by oxidization in the cells of peripheral tissues. This RA generating process is mediated by two key enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AHD). Two well-known isomers of RA, all-*trans* RA and 9-*cis* RA, transactivate retinoid nuclear receptors, RAR and RXR. In addition, another isomer, 9,13-di-*cis* RA, is involved in hepatic fibrogenesis via transactivating RAR. A number of synthetic retinoid have been developed to perform their pharmacological applications including cancer chemoprevention. Acyclic retinoid (NIK 333 or polyprenoic acid) prevented successfully the development (posttherapeutic reccurrence) of HCC in a clinical trial.

apoptosis, differentiation, reproduction and morphogenesis (1). Retinoids modulate inflammatory processes and are required in tissue wound healing. In addition, it is suggested that loss of retinoid activity or responsiveness is linked to carcinogenesis, including the development of hepatocellular carcinoma (HCC) (2). Some of these effects of retinoids are exerted directly via the nuclear receptors (3), but others are mediated indirectly through modulating the activities of cytokines (4). In the present review, we focus upon retinoids' effects on the development of liver fibrosis and HCC and discuss our approach to their therapeutic applications.

Retinoids consist of several molecular species, including retinoic acid (RA) (an active metabolite that binds to its nuclear receptors), retinol (a transport form in the plasma), and retinylesters (storage forms in the tissues) (Figure 1). In addition, large numbers of synthetic retinoids have been developed recently. All natural retinoids originate in the diet either as retinyl esters or provitamin A, carotenoids. Dietary retinyl esters and carotenoids are subjected to a series of metabolic conversions to form retinol in the intestinal mucosa. Retinol is absorbed with other dietary lipids, esterified to retinyl esters and packed in nascent chylomicrons. The chylomicrons are secreted into the lymphatic system and then enter into the circulation. Most chylomicron retinylesters are taken up by the liver, the major storage site of body retinoids. The liver stores retinoids in the form of retinylesters and delivers them as retinol after hydrolysis of the esters to meet the requirement of peripheral tissues. There are specific binding proteins to retinol in the plasma and cells; retinol-binding protein (RBP) and cellular retinol-binding protein (CRBP), respectively. A small portion of dietary retinoids is converted to RA, absorbed through portal vein, and present in the plasma bound to albumin. RA is also biosynthesized in the cells of peripheral tissues and modutates transactivation activities. RA binds to cellular RA-binding protein (CRABP) in the cytoplasm and is further oxidized to an inactive metabolite. RAs exert their biological functions through two distinct nuclear receptors, RA receptor (RAR) and retinoid X receptor (RXR) (3). RAR interacts with both all-*trans*-RA (atRA) and 9-*cis*-RA (9cRA), whereas RXR binds only to 9cRA. 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 several other nuclear receptors. These dimers bind to their respective response elements and subsequently activate or inhibit the expression of their target genes. RAR and RXR bind to a RA response element (RARE) and RXR response element (RXRE), respectively, and regulate transactivation of down-stream genes.

Two types of hepatic cell are known to participate in the retinoid storage and metabolism, hepatic

parenchymal cells (HPCs) and hepatic stellate cells (HSCs) (5). HPCs account for two third of the total liver cells whereas the number of HSCs is only around 5%. However, HSCs play central roles in the storage of retinoids in the normal liver. HPCs take up retinoids from chyromicrons and secrete them as retinol after binding to RBP that is also synthesized by HPCs. The mechanism by which retinoids are transferred between HPCs and HSCs remains unsolved. Moreover, HSCs are the cells that induce fibrosis in the damaged liver (6,7). During the progression of liver fibrosis, HSCs lose retinoid-containing lipid droplets from the cytoplasm, transform into myofibroblast-like cells and start to produce a significant amount of extracellular matrices (ECMs). This transformation is termed "activation" of HSCs. Thus, it is of great interest to understand the relationship between the loss of retinoids and production of ECMs in HSCs. This understanding may lead not only to the elucidation of the mechanism of hepatic fibrosis but also to the development of its therapy.

3.RETINOIDS AND LIVER FIBROSIS

3.1. Controversy of retinoids' effects

Some controversies have been recorded regarding the effects of retinoids on liver fibrosis (8). Some reports have shown anti-fibrotic effects of retinoids, but others suggested a pro-fibrogenic action. For example, administration of retinylpalmitate (a major retinylester in the liver) suppressed experimental hepatic fibrosis in rats produced by either carbon tetrachloride (CCl_4) or by porcine serum (9). In support of this finding, vitamin A deficiency was shown to promote CCl_4-induced liver fibrosis (10). On the other hand, others have reported that vitamin A plus ethanol stimulated the progression of liver fibrosis and cirrhosis in rats (11). Furthermore, the incidence of human hepatic fibrosis was shown to be correlated with the amount of vitamin A intake by patients with vitamin A hepatotoxicity (12). In addition to the *in vivo* effects, accumulating knowledge in molecular biology studies have shown a suppression of some matrix-degrading enzyme gene promoters, including stromelysin and collagenase, by RA (13,14); which may cause a fibrosis-enhancing effect. Thus, conflicting effects have been observed for the effect of retinoids on liver fibrosis depending upon the experimental conditions or upon the molecular species of rertinoids, and it is not known if retinoid loss is required for HSC activation and which retinoids might accelerate or prevent hepatic fibrosis (6). Moreover, it is also uncertain how retinylesters (mostly retinylpalmitate) are lost in HSCs and if retinylesters might be converted to certain retinoid metabolite(s) that may affect HSC activation.

In this chapter, we discuss the ECM-producing effect of RA in HSCs. We suggest the generation of a certain isomer of RA in the process of HSC activation in culture and during liver fibrosis *in vivo*. This RA enhances proteolytic activation of transforming growth factor-β (TGF-β), a strong fibrogenic cytokine, by up-regulating the plasminogen activator (PA)/plasmin system. Active TGF-β in turn stimulates its own synthesis and enhances ECM production by HSCs.

3.2. RA-induced TGF-b activation

We have shown that a stable analog of RA exacerbated liver fibrosis by enhancing the function of TGF-β in the fibrosis model induced by porcine serum, in which pure fibrosis is generated without causing hepatic inflammation or parenchymal necrosis (15). In this model, HSCs are the cells that mainly produce TGF-β. We have demonstrated that the ECM-producing effect of RA is mediated via activating a fibrogenic cytokine, TGF-β.

TGF-β is a major cytokine implicated in the pathogenesis of liver fibrosis and cirrhosis (16). TGF-β stimulates HSCs to transform into myofibroblast-like cells, enhances their production of ECM proteins, and suppresses the degradation of the ECM (6,7). Three subtypes of TGF-β (TGF-β 1, -β 2 and -β 3), whose biological properties are nearly identical, are found in mammals (17). TGF-β is synthesized and secreted in a biologically latent form (latent TGF-β) which needs to be activated to acquire binding capacity to its cognate receptors and to perform biological activities (4). Activation releases the 25-kDa TGF-β homodimetric molecule by proteolysis from the latent large complex. Plasmin-mediated activation occurs under physiological conditions such as in the co-cultures of vascular endothelial and smooth muscle cells (18). In this system, activation occurs on the cell-surface by plasmin generated from serum plasminogen by the action of PA (19). It has been reported that RA enhances the production of both PAs in many cell types (20). In bovine vascular endothelial cells, the elevation of cell surface PA/plasmin levels by RA causes the formation of active TGF-β; this TGF-β subsequently mediates some of the effects of RA on the endothelial cells (21,22).

We have demonstrated that RA also stimulates the formation of active TGF-β in rat HSC cultures via plasmin-mediated proteolytic cleavage of latent TGF-β on the cell surface (15) (Figure 2). The resultant active TGF-β stimulates its own synthesis, resulting in the generation of a considerable amount of TGF-β. Active TGF-β enhances collagen production and suppresses collagenase activities in HSC cultures, leading to the accumulation of ECMs. In keeping with the culture experiments, an *in vivo* liver fibrosis model induced by porcine serum also showed increased concentration of TGF-β in the liver as well as the exacerbation of hepatic fibrosis by RA administration. However, interestingly, RA-treatment alone (without porcine serum) does not cause an increase in hepatic TGF-β nor liver fibrosis. Thus, RA does not directly cause liver fibrosis by itself, but rather exacerbates the fibrosis induced by the other stimulus such as porcine serum. In other words, HSCs may need to be activated by a stimulus and to produce an increased amount of TGF-β before they become sensitive to RA-treatment.

Because the effect of RA on TGF-β activation had been examined by administering exogenous RA to the animals (15), we further examined the effect of endogenously produced RA. It is well known that retinylpalmitate contents, a storage form of retinoids, decrease in HSCs with the development of fibrosis (5).

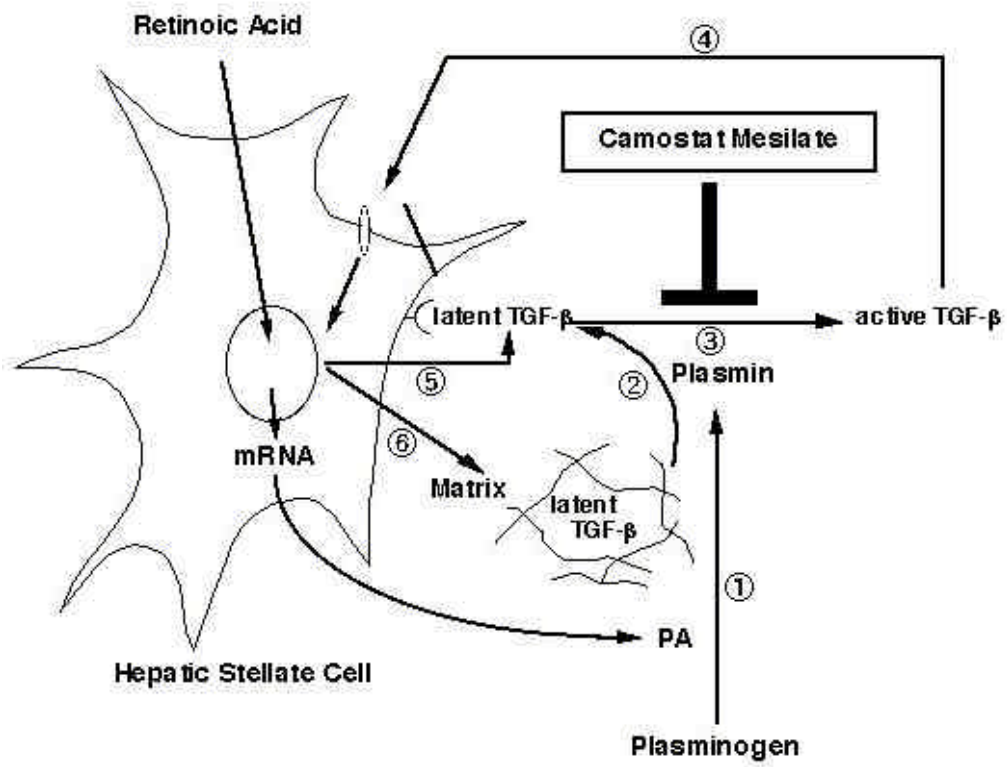

Figure 2. Schematic illustration of RA-induced promotion of liver fibrosis via TGF-β activation, and its therapeutic control by a protease inhibitor, camostat mesilate. RA up-regulates PA/plasmin levels in HSCs (1) and thereby elaborates the release (2) and the activation (3) of latent TGF-β on the cell surface. The active TGF-β generated then stimulates the activation (or transformation) of HSCs (4), promotes TGF-β's own synthesis (5) and enhances the production of ECM (6), resulting in a cycle of TGF-β/ECM overexpression and an exacerbation of liver fibrosis. Camostat mesilate inhibits this proteolytic activation of TGF-β and thereby prevents liver fibrosis. (From reference 15, with permission).

This implies the possibility that the loss of retinylpalmitate in the fibrotic liver might be ascribed, in part, to the result of conversion of retinylpalmitate to some other metabolites, including RA. In support of this hypothesis, we have discovered an increase in 9,13-di-*cis*-RA generation, a major isomer of RA, in rat fibrotic livers induced by porcine serum (23) (Figure 3). Because 9,13-di-*cis*-RA is a major product arising from the *in vivo* isomerization of 9-*cis*-RA (24,25) and has been suggested to be an indicator of pre-existed 9-*cis*-RA (26), the elevation of hepatic 9,13-di-*cis*-RA concentration may suggest that 9-*cis*-RA might be generated during the development of fibrosis. In addition, we have found that 9,13-di-*cis*-RA itself can induce PA and thereby activate latent TGF-β via a plasmin-dependent manner, resulting in enhanced TGF-β biosynthesis (27). This biological action of 9,13-di-*cis*-RA seems to be mediated by a nuclear RA receptor, RARα.

However, a contradictory observation has also been reported, showing that RA content as well as its signaling was diminished in the fibrotic liver induced by cholestasis (28). The reason for this discrepancy still remains to be elucidated; however, the difference in the animal models (immunologic stimulus by porcine serum vs. cholestatic stimulus by bile duct ligation) might influence the retinoid absorption and metabolism in distinct manners, resulting in the difference in the experimental outcome. For instance, lack of bile in the intestine due to bile duct ligation may interfere with the absorption of retinoids, leading to rapid depletion of liver retinoids content, including RA.

3.3. Possible mechanisms to explain the controversy

Based on our present and previous results, we hypothesize the following two possibilities to explain the controversy regarding the effects of retinoids on liver fibrosis (8). First, the overloading of retinylesters, storage forms of retinoids that does not have biological activity by themselves, to HSCs may cause the excess accumulation of lipid droplets in the cytoplasm and would interfere with the protein synthesis including ECMs. On the contrary, administration of RA that has transactivating activity would directly modulate specific gene expression by the interaction with RARs and stimulate ECM production. Second, the apparent discrepancy between hepatic fibrosis models might be ascribed to the presence and absence of inflammation in the liver. Because RA is known to suppress the inflammation and accelerate scar formation in

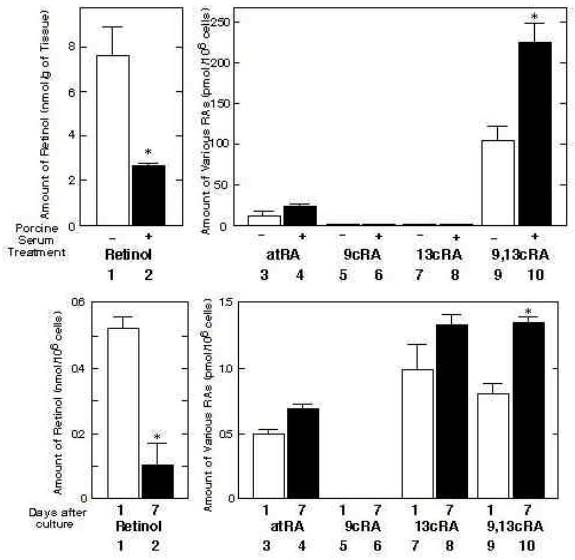

Figure 3. Increases in 9,13-di-*cis* RA levels in the fibrotic liver and activated HSCs in culture. *Panel* A, Liver fibrosis was induced in rats by continuous intraperitoneal injections of porcine serum for 12 weeks. The hepatic concentrations of retinol (columns 1 and 2), all-*trans* RA (atRA; columns 3 and 4), 9-*cis* RA (9cRA; columns 5 and 6), 13-*cis* RA (13cRA; columns 7 and 8), and 9,13-di-*cis* RA (9,13dcRA; columns 9 and 10) were determined by high performance liquid chromatography (HPLC). Odd numbers: control rats administered saline; even numbers: fibrotic rats administered porcine serum. *Panel* B, Retinoids were extracted from primary rat HSCs cultured for either 1 day or 7 days and were determined by HPLC. HSCs remained quiescent on day 1 and were spontaneously activated (transformed) during 7-days culture. Odd numbers: HSCs cultured for 1 day; even numbers: HSCs cultured for 7 days. For A and B, Each value represents the average ± SD (n=6 for A and n=5 for B). Asterisks represent significant differences ($p<0.05$). Note that 9,13-di-*cis* RA increased significantly in both fibrotic liver and activated HSCs in culture. (From reference 23, with permission).

the wounded tissues, it may inhibit fibrosis indirectly via modulation of hepatic inflammation. These anti-inflammatory and subsequent anti-fibrotic effects of RA were seen in the CCl4-induced cirrhotic liver in rats, which is accompanied with parenchymal necroinflammation (29). In contrast, RA may also possess a potential to stimulate hepatic fibrosis specifically when it acts directly on HSCs. This effect may be observed in the pure fibrosis model such as the one induced by porcine serum, in which hepatic inflammation is absent (15,23). In the latter condition, RA seems to regulate transactivation directly in HSCs, and induces PA/plasmin-dependent activation of TGF-β.

3.4. Protease inhibitor

Our studies demonstrate a fibrogenic aspect of RA in the liver via enhancing proteolytic activation of TGF-β. These studies may provide a clue for a novel therapy against liver fibrosis. Because the induction of TGF-β by RA in HSCs was initiated by the activation of latent TGF-β on the cell surface, this TGF-β activation can be a primary target for therapeutic strategies, and inhibitors of PA/plasmin could be potent anti-fibrogenic agents. In fact, the plasmin/TGF-β activation cascade has been shown to occur in human hepatic fibrosis (30). Interestingly, in previous studies, protease inhibitors and an inhibitor of cell surface plasmin have been used as cytoprotective agents to prevent hepatic necrosis (31).

This idea led us to examine which protease inhibitors would be effective in suppressing TGF-β generation, in cultured HSCs (32). Most protease inhibitors that we tested in cultured HSCs suppressed activation and generation of TGF-β to some extent. Similar results were obtained with HSCs stimulated by either RA or basic fibroblast growth factor (basic FGF), reagents that increase endogenous plasmin levels in the cells. Among the compounds examined, we selected one chemical, camostat mesilate (Figure 4), and examined its effect on the phenotypic changes (or transformation) of HSCs. Currently, camostat mesilate is used clinically for the therapy of pancreatitis and reflux esophagitis, and its safety is widely confirmed from clinical experiences (33,34). When rat primary HSCs were cultured with camostat mesilate, the compound suppressed cell surface plasmin levels and thereby reduced the activation of TGF-β as well as its auto-induction. Moreover, the compound inhibited morphological changes of HSCs (for example, loss of lipid droplets from the cytoplasm), down-regulated the expression of α-smooth muscle actin (αSMA), a marker of activated HSCs, and inhibited cellular proliferation (35) (Figure 5). The compound was also highly effective in suppressing collagen production. Thus, camostat mesilate inhibited not only the generation of active TGF-β but also transformation of HSCs, resulting in a marked suppression of ECM production.

3.5. Prevention of hepatic fibrosis

These results suggest that the protease inhibitor may be useful also *in vivo*, suppressing the generation of TGF-β in the liver and subsequent development of hepatic fibrosis. We next examined the effect of camostat mesilate using an *in vivo* model of liver fibrosis induced by porcine serum. Oral administration with camostat mesilate suppressed hepatic TGF-β mRNA expression and HSC activation morphologically when examined by immunohistochemistry of αSMA and by electron microscopy. Camostat mesilate appeared to prevent porcine serum-induced fibrosis, as assessed by hydroxyproline content and histologic observation, without causing any obvious adverse effects at the same dose range administered to humans (35). Plasmin has been believed to be anti-fibrogenic because it dissolves ECM directly or indirectly via activating pro-metalloproteinases (36,37). Expression of PA increases at the early stage of fibrosis; however, the expression of PA inhibitor-1 (PAI-1) is enhanced and overwhelms PA expression in the late phase, leading to a decrease in net fibrinolytic potential (38,39). We have reported that in a hepatic fibrosis model induced by porcine serum, plasmin exerts a fibrogenic effect in the early stage because it releases latent TGF-β from

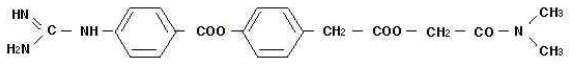

Figure 4. Chemical structure of camostat mesilate (MW: 494.53 kD). Camostat mesilate, a broad-spectrum protease inhibitor, is widely used clinically for the treatment of chronic pancreatitis and reflux esophagitis. (From reference 35, with permission).

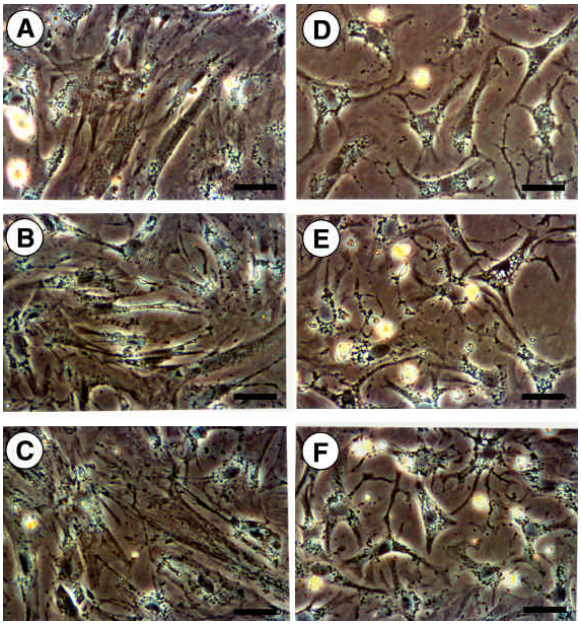

Figure 5. Inhibition by camostat mesilate of activation of primary rat HSCs in culture. Primary rat HSCs were cultured for 7 days in the absence or presence of camostat mesilate, plasminogen, recombinant TGF-β1, or their combination. Cell structure was observed under a phase-contrast microscope. *Panel* A, no reagent (control); *panel* B, plasminogen-included; *panel* C, recombinant TGF-β1; *panel* D, camostat mesilate; *panel* E, plasminogen + camostat mesilate; and *panel* F, recombinant TGF-β1 + camostat mesilate. Scale bar, 25 μm. Camostat mesilate suppressed spontaneous activation of HSCs (compare *panels* A and D), and also in plasminogen-stimulated culture (compare *panels* B and E) as well as in recombinant TGF-β1-treated cells (compare *panels* C and F). Since HSC activation is mainly stimulated by TGF-β, the results suggest that camostat mesilate inhibits both plasmin-mediated activation of TGF-β (*panel* E) and the function of active TGF-β itself (*panel* F), and thereby suppressed HSC activation. (From reference 35, with permission).

surrounding matrices and converts it to the active form (15,35). Moreover, we have shown that hepatic plasmin activity increases along with the development of hepatic fibrosis. Similarly to the *in vitro* data, camostat mesilate suppressed the increases in hepatic plasmin levels and TGF-β production, probably by inhibiting TGF-β activation in HSCs (35). Based upon these results, we suggest that camostat mesilate may inhibit both proteolytic release and activation of latent TGF-β *in vivo*, leading to a reduction in the hepatic TGF-β content. The result appears to link to the maintenance of HSCs in a quiescent phenotype, and to the prevention of hepatic fibrosis.

Since camostat mesilate is a broad-spectrum protease inhibitor, the result might also suggest that this protease inhibitor blocked the activity of not only plasmin but other unknown proteases capable of generating TGF-β. To examine this possibility, we enhanced hepatic plasmin levels by *in vivo* transfection with the urokinase-type PA (uPA) gene and examined if this treatment to enhance hepatic plasmin levels neutralizes the anti-fibrotic effect of camostat mesilate (35) (Figure 6). In fact, up-regulation of the plasmin levels abolished the suppressive effects of camostat mesilate on TGF-β levels and on fibrosis. We therefore conclude that anti-fibrotic effect of camostat mesilate is dependent upon its anti-plasmin activity. However, these results do not exclude the possible use of other protease inhibitors that suppress TGF-β activation by inhibiting other proteases than plasmin.

It may be possible that camostat mesilate might also suppress matrix degradation via a direct and/or indirect inhibition of matrix degrading enzymes, such as matrix metalloproteinase 2 (36,37), and therefore, the compound might be ineffective in the treatment of established hepatic fibrosis. However, we have confirmed the therapeutic (or fibrosis-reducing) effect of camostat mesilate by administering the compound to rats after hepatic fibrosis was established by porcine serum injection (35). Thus, the drug may have potential not only to prevent but also to reduce hepatic fibrosis.

An advantage of camostat mesilate is that it can be administered orally. This advantage will be particularly favorable for the long-term treatment of patients with chronic liver diseases. There have been several reported methods to inhibit TGF-β activity *in vivo*, including injections with anti-TGF-β antibody (40) and infusion with adenoviral vectors expressing either a truncated TGF-β receptor (41) or a soluble TGF-β receptor (42), both of which trap active TGF-β and sequester it from the wild-type cell surface receptors. Although these methods hold some promise, the chronic use of these latter agents may not be focused in present technical challenges.

3.6. Perspective

It is of interest to test the synergistic effect of a combination of camostat mesilate with other anti-fibrotic agents. In addition, as TGF-β is known to be involved in fibrogenesis not only in the liver but also in other organs, such as the lung, kidney and skin, we expect that camostat mesilate might also be effective in fibrogenesis in these organs.

In addition, another important function of TGF-β is to suppress hepatic parenchymal regeneration (17) and function (43). Thus, the protease inhibitor might also be useful for the therapy of impaired liver regeneration often encountered after surgical resections of cirrhotic livers. We have extended our idea and have obtained preliminary results, supporting such beneficial effects of the compound.

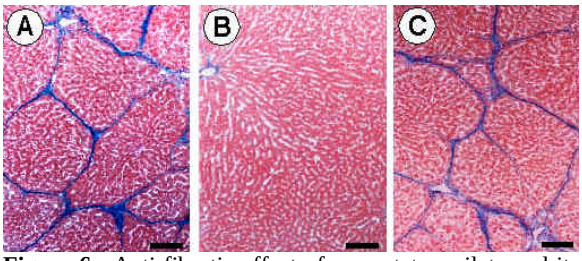

Figure 6. Anti-fibrotic effect of camostat mesilate and its neutralization by overexpressing urokinase type PA (uPA) in the liver. Hepatic fibrosis was induced in rats by continuous intraperitoneal injections with porcine serum for 16 weeks. Rats were also fed either a control diet or a diet containing camostat mesilate. In one half of the porcine serum + camostat mesilate-treated animals, uPA was lipofected *in vivo* starting from 8 weeks until the end of the experiment. Liver tissues were stained by Azan-Mallory method. *Panel* A, porcine serum-treated and vacant vector-transfected rat; *panel* B, porcine serum-treated, camostat mesilate-administered, and vacant vector-transfected rat; *panel* C, porcine serum-treated, camostat mesilate-administered, and uPA expressing vector-transfected rat. Scale bar, 100 μm. Camostat mesilate suppressed liver fibrosis (compare *panels* A and B). This inhibition of hepatic fibrosis by camostat mesilate was abolished by enhancing hepatic plasmin levels through inducing the uPA gene in the liver (compare *panels* B and C), suggesting that the anti-fibrotic effect of camostat mesilate is mediated by the suppression of plasmin activity. (From reference 35, with permission).

The compound restored hepatic regenertion after partial hepatectomy in rats, which had been suppressed by the action of TGF-β secreted from HSCs by the stimulation of lipopolysaccharide (LPS) (Akita K, et al., unpublished observations). In this system, latent TGF-β was probably activated by protease(s) other than plasmin, since LPS completely suppressed cell surface plasmin levels. Such protease(s) seems to include plasma kallikrein whose activity is also inhibited by camostat mesilate. Thus, activation of latent TGF-β is a key event for the pathogenesis of hepatic fibrosis as well as impaired liver regeneration, and may be an important target for the therapy of such pathologic states. Because impaired liver regeneration is crucial for the prognosis not only of cirrhotic patients after partial hepatectomy but also of patients with fulminant hepatic failure, development of drugs to restore regeneration, including comostat mesilate, may be of great importance to improve the clinical outcome of a series of human disorders.

4. RETINOIDS AND LIVER CANCER

4.1. Chemoprevention of HCC

HCC has become one of the most frequent cancers in the world. Since HCC is closely related to hepatitis viral infections and commonly arises in the liver with chronic inflammation, HCC is more often seen in areas with high hepatitis viral infections including East Asia (44). In addition, the incidence of HCC has been rising recently in countries including the United States (45,46) and Western Europe (47,48), where the number of people with hepatitis viral infections has also been increasing. The annual incidence of HCC reaches approximately 3% in type B (HBV)- and 7% in type C (HCV)- hepatitis virus-infected cirrhotic patients in Japan (49,50). Moreover, the annual incidence rises to approximately 20-25% in cirrhotic patients who underwent potentially curative treatment of the primary HCC (*i.e.*, surgical resection, percutaneous ethanol injection therapy and ablation therapy) (51-53). In fact, the recurrence rate after 5 years of potentially curative treatment reaches as high as 70% or more (54). Importantly, at least one third of the posttherapeutic recurrence is the *de novo* cancer due to multicentric carginogenesis, but not intrahepatic recurrence or metastasis from the original lesion (55). Thus, despite extensive clinical advances in the therapy of HCC, this high incidence of posttherapeutic recurrence is a major cause of a limited 5-year survival rate (approximately 40%) after the curative treatment (56). Therefore, future advances in medical technologies in both the early detection and therapy of HCC may not largely serve to further improve the therapeutic outcome of HCC, rather a new strategy to prevent posttherapeutic recurrence of HCC may be required for this purpose.

So far, clinical experience has demonstrated some strategies to suppress the development of HCC, including HBV vaccination (57), interferon (IFN)-α and -β (58-61), a Japanese herbal medicine, TJ-9 (62), glycyrrhizin (63) and our retinoid analog, acyclic retinoid (64,65) (for review, see reference 66). Eradication of HBV by vaccination reduced the incidence of HCC in children in Taiwan (57). However, vaccination against HCV, which is now more common than HBV, is not available, so other preventive methods are required for HCV-infected patients. IFN-α and -β are the only drugs for the eradication of HCV and is widely used in HCV-infected patients with chronic liver diseases (67). IFN induces a continuous clearance of HCV-RNA in 15-35% of treated patients (68), and suppresses hepatic necroinflammation in some cases even when it fails to eradicate HCV, resulting in the delay in progression toward cirrhosis. In either case with or without successful HCV eradication, IFN is thought to reduce the incidence of HCC (58-61), although not all studies are positive in this regard. Glycyrrhizin also suppresses serum aminotransferases and thereby prevents tumor development, although it does not have antiviral activity against either HBV or HCV (63). Thus, suppression of hepatic necroinflammation by these drugs may serve to prevent hepatocarcinogenesis. IFN and glycyrrhizin may belong to a category of immunopreventive agents, functioning as biological response modifiers. On the other hand, acyclic retinoid is a member of chemopreventive agents, since the retinoid seems to act directly on (pre)malignant cells without modulating hepatic necroinflammation as will be discussed in the later section. IFN and glycyrrhizin have been shown to successfully prevent the occurrence of primary HCC in cirrhotic patients, and acyclic retinoid has been shown to suppress the posttherapeutic recurrence after curative treatment of previous tumors. In addition, more specialized methods to prevent the recurrence of HCC have been reported recently;

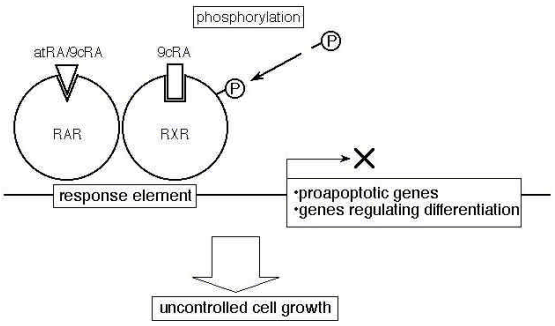

Figure 7. Retinoid-refractoriness in HCC cells. RXRα, a retinoid nuclear receptor, is post-translationally modified by phosphorylation in the cancer cells leading to a loss of its transactivating activity. This phosphorylation is mediated by MAP kinases. The failure in inducing its down-stream genes that regulate cellular proliferation, apoptosis and differentiation may lead the cells to uncontrolled growth. **X**: interruption. (From reference 75, with permission).

intra-arterial injection with radiolabeled lipiodol that accumulates in invisible tumor tissues for weeks (69), andadoptive immunotherapy (70). However, these two therapies may have limitations for wide clinical applications because of their technical complexity.

Here, we focus upon acyclic retionid, and discuss its clinical benefits and molecular mechanisms.

4.2. Retinoid refractoriness

We have shown a depletion of retinoid in tumor tissues in both experimental and clinical HCC (71). Absence of HSCs, retinoid-storing cells in the liver, is a major cause of such retinoid-depletion in the tumors. Moreover, a rapid metabolism of retinoid into an inactive metabolite may well participate in such depletion (71). We have shown a rapid conversion of retinol to anhydroretinol, an inactive retinol metabolite that can not be metabolized to RA, an active ligand of retinoid nuclear receptors. Since there have been several reports showing chemopreventive effects of retinoids on liver cancer in experimental studies (72), it seems conceivable that retinoid deficiency may promote tumorigenesis of the liver. In a clinical study, we have suggested that the enhanced loss of retinoid in the liver of cirrhotics with viral infection plus alcohol abuse may be linked to the accelerated incidence of HCC (73). Thus, retinoid-depletion might well be involved in hepatocarcinogenesis.

In addition to retinoid-depletion, we have also shown a malfunction of retinoid nuclear receptor in HCC cells. Retinoids exert their biological functions through two distinct nuclear receptors, RAR and RXR (3). It is suggested that both RAR and RXR are involved in hepatocarcinogenesis. For example, RXRα is reported to bind to the enhancer element of hepatitis B virus and modulate viral replication (74). Among these receptors, RXRα is most abundant in the liver and highly expressed in HCC cells. Very recently, we have found that full-length RXRα protein is phosphorylated and accumulated in both surgically resected HCC tissues and HCC cell lines (75). In contrast, RXRα is unphosphorylated and is rapidly broken into smaller peptides in normal liver and normal hepatocyte cultures. Phosphorylation of RXRα takes place both at serine 260 and threonine 82 residues, consensus sites of mitogen-activated protein kinase (MAP kinase) that plays a significant role in such phosphorylation. Phosphorylated RXRα loses its transactivating activity, which seems to correlate with enhanced proliferation of HCC cells. In support of this growth-promoting effect, transfection with phosphomimic mutant RXRα (mutation with serine 259 and 260 to aspartate) enhances cellular proliferation even in normal hepatocyte cultures. Although we have not fully identified the down-stream gene(s) of RXRα that regulate cellular proliferation yet, these observations may suggest that malfunction of phosphorylated RXRα is linked to the aberrant growth of HCC (Figure 7). Moreover, phosphorylated RXRα is sequestered from proteolytic degradation via ubiquitin/proteasome-mediated pathway, resulting in an accumulation of non-functioning phosphorylated RXRα in the tumors (Adachi S. et al., in submission). Therefore, phosphorylated RXRα might act as a dominant-negative receptor that interferes with transactivating function of unphosphorylated normal receptor. Similarly, a dominant negative RA receptor has been reported in acute promyelocytic leukemia (APL) (76). In APL, a characteristic chromosome t(15:17) translocation results in a fusion between the RARα gene and a region referred to PML. This chimera protein, PML-RAR renders promyelocytes refractory to RA, functioning as a dominant negative receptor, and thereby sequesters the cells from normal differentiation. Our observations also suggest that in HCC tissues and cells not only retinoid-depletion but also malfunction of phosphorylated retinoid nuclear receptor may be involved in the development of the cancer.

4.3. Acyclic retinoid

Acyclic retinoid (all *trans*-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentanoic acid), or NIK 333 (Nikken Pharmaceutical Co., Tokyo, Japan) (Figure 1), inhibits experimental liver carcinogenesis (71,72) and induces the apoptosis of human hepatoma-derived cell lines (77). In spontaneous tumor-bearing C3H/HeNCrj mice, liver tumors develop genetically in more than 90% of animals after 60 weeks. Acyclic retinoid suppressed significantly the development of liver tumors when given orally (71). In an experimental rat model treated with 3'-methyl-4-dimethylaminoazobenzene (3'MeDAB), oral administration of acyclic retinoid also suppressed the appearance of oval cells, progenitor cells of HCC as well as cholangiocelluler carcinoma (CCC), in the early stage of carcinogenesis, and the subsequent development of both HCC and CCC in the late stage (Tanaka T, and Sano T, et al., unpublished observations). In addition, acyclic retinoid suppresses dimethylnitrosamine (DEN)-induced HCC in rats, when administered in the promotion phase (Tanaka T, and Sano T, et al., unpublished observations). Thus, this retinoid seems to exert preventive effects on hepatocarcinogenesis in both early and late phases. There was no serious adverse effect in either model except mild hypertriglyceridemia.

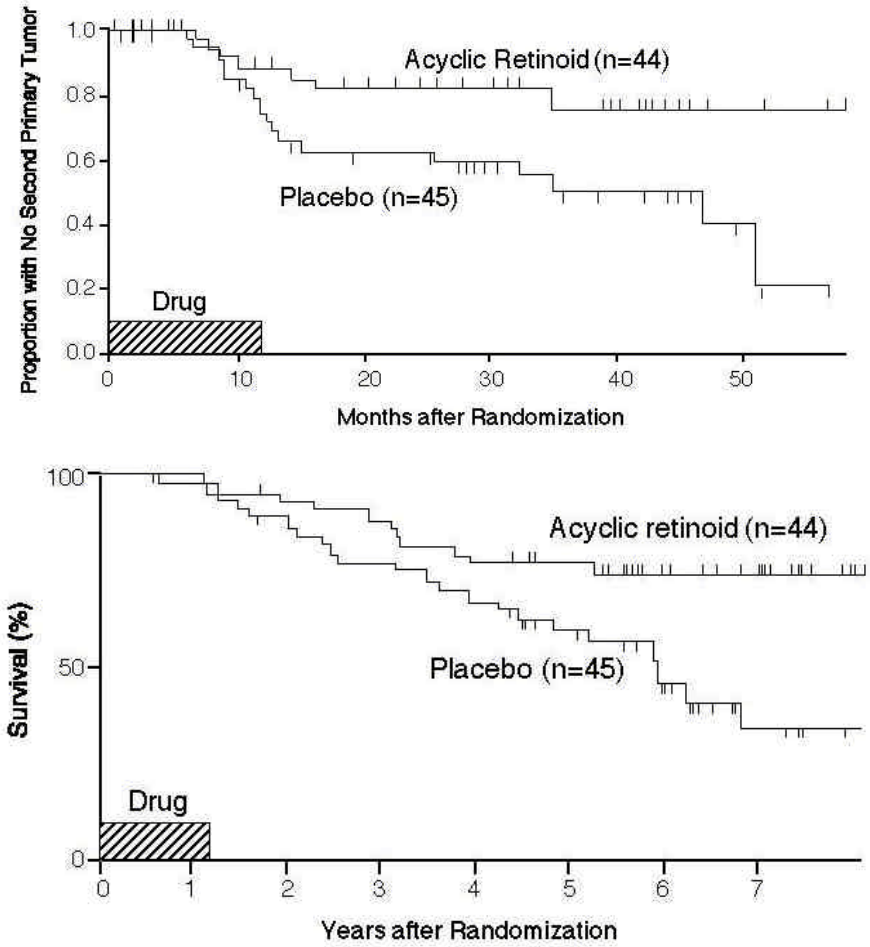

Figure 8. Prevention by acyclic retinoid of posttherapeutic recurrence of HCC in a double-blind clinical study. After potentially complete removal of preceding HCC, cirrhotic patients were given acyclic retinoid or placebo orally for 12 months and were examined by diagnostic imaging (ultrasonography and computed tomography) in every three months. Kaplan-Meier estimates of the propotion of patients without second HCC (*panel* A) and with survival (*panel* B). Acyclic retinoid not only prevented posttherapeutic recurrence of HCC but also improved the survival. The estimated 6-year survival was 74% in the retinoid group and 46% in the placebo group. (From references 64 and 65, with permission).

After confirming such beneficial actions and the absence of adverse effects in experimental animals, we extended the study, examining the chemopreventive effect of acyclic retinoid on HCC in a double-blind and placebo-controlled clinical study (64,65). We selected patients with HCC who underwent potentially curative treatments (*i.e.*, surgical resection and percutaneous ethanol injection therapy) and did not exhibit any abnormalities that suggest tumor recurrence for a 3-months interval after the initial therapy. Oral administration of acyclic retinoid (600 mg daily) for only 12 months significantly reduced the incidence of posttherapeutic recurrence as compared to the placebo group (adjusted relative risk, 0.31; mean follow-up period, 38 months) (64) (Figure 8A). Moreover, the survival rate was also significantly improved by the compound after a median observation period of 62 months in the follow-up study (adjusted relative risk, 0.3) (Figure 8B) (65). Such long-term effects of acyclic retinoid seems surprising because patients were given the retinoid for only 12 months and did not receive any other additional therapy thereafter.

In that clinical trial, serum lectin-reactive α-fetoprotein (AFP-L3), which indicates the presence of transformed hepatic cells in the remnant liver, disappeared in the acyclic retinoid group after a 12-months administration (78) (Figure 9). Because clinical experience suggests that serum AFP-L3 predicts the development of HCC several months before its detection by diagnostic imaging, AFP-L3 is believed to indicate the presence of unrecognizable cancer cell in the liver (79). Our observation suggests that AFP-L3-producing clones were eliminated by acyclic retinoid from the remnant liver. In contrast, spontaneous reduction in serum AFP-L3 levels was not observed in the placebo group. Thus, we suggest that acyclic retinoid removed premalignant or latent malignant clones that produce AFP-L3 from the remnant liver. Moreover, acyclic retinoid suppressed the appearance of serum AFP-L3 in patients whose AFP-L3 levels were below the detection range at the entry of the clinical trial, whereas the number of patients whose serum AFP-L3 appeared *de novo* was significantly increased in

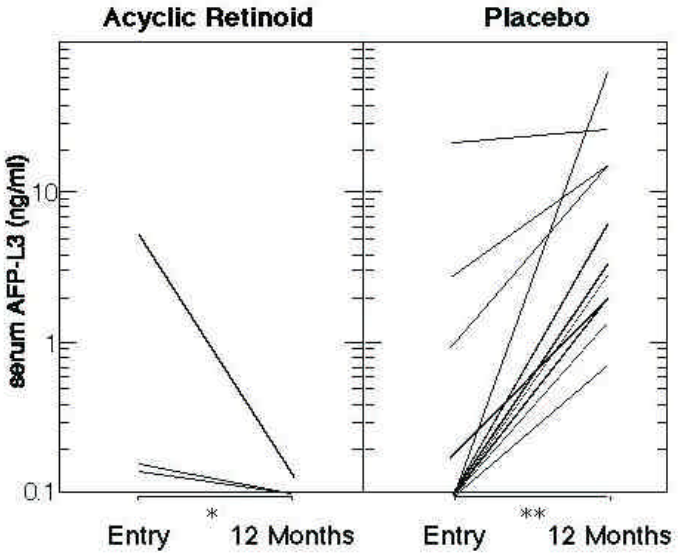

Figure 9. Deletion of AFP-L3-producing cell clones from the liver by acyclic retinoid. Serum AFP-L3 levels were monitored in the patients shown in Figure 8 both at the entry and after 12 months of the drug administration. Serum AFP-L3 levels remained under the detection limit in 16 patients with retinoid-administration (*) and in 9 patients with placebo (**). Serum AFP-L3 disappeared after acyclic retinoid-administration in patients who had detectable serum levels at entry, and acyclic retinoid also suppressed the appearance of AFP-L3 in patients whose levels were under detection limit at the entry, suggesting "clonal deletion" and "clonal inhibition" of AFP-L3-producing transformed cells from the remnant liver. (From reference 78, with permission).

the placebo group. From these results, we have proposed the new concepts of cancer chemoprevention, "clonal deletion" and "clonal inhibition", for the respective cases (80). These new concepts may explain the reason why only a short-term administration (12 months) of acyclic retinoid has exerted a long-term suppressive effect on the development of HCC for several years even after terminating the administration. Once (pre)malignant clones are deleted, it would take at least several years for the development of *de novo* cancer in the cirrhotic liver.

4.4. Clonal deletion

"Clonal deletion" therapy suggests a removal of latent malignant (or premalignant) cells that are invisible by diagnostic images from the liver (Figure 10). Because of a high incidence of HCC in cirrhotic patients, it may well be likely that some latent malignant (or premalignant) cells exist in the cirrhotic liver under the detection limit of imaging modalities. Acyclic retinoid might remove such clones from the liver and thereby reduce the incidence of a second HCC. Because the removal of such (pre)malignant clones could be recognized as a therapy and more than disease prevention, these ideas may allow us to place chemoprevention in the same category as chemotherapy (81).

In general, two possible mechanisms are widely accepted for such removal of transformed cells: cell death (or apoptosis) and differentiation induction. These two mechanisms may work independently in some cases, but work concurrently in other systems. One of the typical examples is the RA therapy against APL (82). RA administered exogenously restores the function of the chimera receptor, PML-RAR, and induces the differentiation of APL cells into granulocytes. Once differentiated, granulocytes restore their normal function and terminate their lives by apoptosis. This phenomenon is termed "terminal differentiation". Similar mechanisms are hypothesized in the chemoprevention of several cancers. We have shown the induction of both apoptosis (77) and differentiation (83) by acyclic retinoid in HCC cells. So far, only limited information is available regarding differentiation-induction in HCC cells. We have reported the up-regulation of albumin with the down-regulation of AFP expressions with acyclic retinoid administration (83). However, these observations might not be enough to draw conclusions. Further investigation is required to show the phenotypic changes of HCC cells, including morphological changes or sequential genetic alterations that are typical for mature hepatocytes. In contrast, induction of apoptosis in HCC cells by acyclic retinoid has been shown more clearly further elucidated. Kojima et al. have proposed an involvement of tissue transglutaminase in the RA-induced apoptosis in APL cells (Kojima S, unpublished observation). There have been several reports showing an induction of tissue transglutaminase in RA-induced apoptotic cells (84). However, the function of tissue transglutaminase in apoptosis has not been fully demonstrated yet. We have found that acyclic retinoid-induced apoptosis in HCC cell lines is also dependent not only on caspases but also on tissue transglutaminase (Sano T and Kojima S, unpublished observations). Acyclic

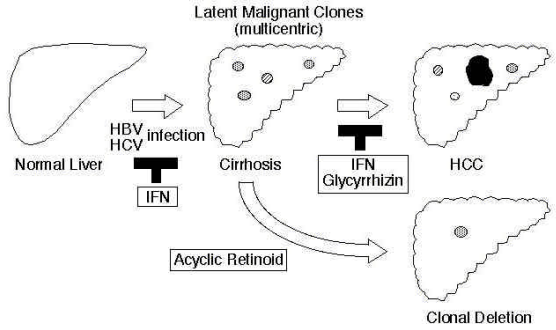

Figure 10. The concept of "clonal deletion" therapy. A high incidence of HCC in cirrhotic patients strongly suggests the presence of minute or latent malignant cells in the liver that are invisible by diagnostic imaging. It is suggested that there may be multiple (pre)malignant clones in the cirrhotic liver due to multicentric carcinogenesis (indicated by dotted and shaded circles). Therefore, eradication of such transformed clones ('clonal deletion') may be recognized as the therapy instead of prevention. Our clinical experience suggests a deletion of AFP-L3-producing transformed clones from the liver by acyclic retinoid therapy. Once such clones are deleted, the preventive effect on HCC (indicated by a larger closed circle in HCC liver) would last at least for several years without continuous administration of the retinoid. IFN suppresses the development of cirrhosis by eradicating HCV or by reducing hepatic necroinflammation even when IFN fails to eradicate the virus. Glycyrrhizin also suppresses hepatic necroinflammation although the drug does not have anti-viral activity. IFN and glycyrrhizin prevent the development of HCC by acting as biological response modifiers. Acyclic retinoid belongs to chemopreventive agents while IFN and glycyrrhizin belong to immunopreventive ones. Since their mechanisms of cancer prevention are different, a synergistic effect might be expected by the combined use of IFN and acyclic retinoid in future clinical use. (From reference 66, with permission).

retinoid activates both caspases and tissue transglutaminase simultaneously. However, in a certain condition, this acyclic retinoid-induced apoptosis is not inhibited by caspase inhibitors, but is specifically suppressed by antisense oligo DNA specific to tissue transglutaminase. and its underlying molecular mechanism(s) has been The HCC cells treated with acyclic retinoid showed typical features of apoptotic changes including both chromatin condensation when examined by electron microscopy as well as DNA ladder formation. We therefore speculate that both tissue transglutaminase and caspase work simultaneously.

4.5. Perspective

"Clonal deletion" therapy has already been theoretically proposed as a mechanism of cancer chemoprevention (85-87), and is strongly supported by our clinical experience of HCC (78,80). The benefit of clinical application of this new concept may place chemoprevention in the same category as chemotherapy, which would make cancer prevention more easily acceptable in the clinical field. In addition to the prevention of posttherapeutic recurrence, attention should be paid to the prevention of primary HCC in cirrhotic patients. As discussed above, it is widely recognized that there may be minute or latent malignant cells in the cirrhotic livers of such patients. Therefore, even when the tumors can not be detected by diagnostic imaging, there seems to be good reason to treat such patients with "clonal deletion" therapy.

Since the preventive mechanisms are distinct between immunoprevention by IFN and glycyrrhizin and chemoprevention by acyclic retinoid, their combination could have synergistic effects. In fact, very recently, we have shown a synergistic effect of IFN and acyclic retinoid on the growth suppression of several human HCC-derived cell lines (Obora A. et al., unpublished observations). This effect seems to be mediated in part by the up-regulation of type I IFN receptor and STAT-1, a signal transducing molecule, in the cancer cells by the retinoid. Moreover, some novel genes associated with the anti-proliferative effect of a combination of IFN and RA have recently been identified (88).

5. CONCLUSION

Removal of the causes of liver disease is undoubtedly the most fundamental therapy. However, unfortunately, failure in eradicating hepatitis viruses is often encountered in clinical experiences. Thus, alternative ways to inhibit disease progression towards cirrhosis is required. In this sense, prevention of hepatic fibrosis is an important strategy that serves to restore liver function. In addition, hepatic fibrosis itself is reported to be a risk factor for HCC; *i.e.*, HCC is more likely to develop in the liver with severe fibrosis (61). Thus, the reduction in fibrosis may also serve to reduce the risk of HCC. For such a purpose, a number of approaches have been attempted, including our protease inhibitor (35).

Moreover, the prognosis of cirrhotic patients associated with hepatitis viral infection is greatly influenced by the development of HCC. Therefore, every attempt should be performed to prevent HCC in such a high-risk group. Again, it should be emphasized that both IFN and acyclic retinoid not only have successfully prevented HCC, but also have improved the survival rate (65). It thus appears reasonable to recognize IFN and acyclic retinoid as therapeutic drugs rather than preventive ones, and they should be used to eliminate latent (pre)malignant clones from the diseased liver with chronic inflammation. Acyclic retinoid is now being employed in a clinical trial at National Cancer Center in Tokyo, Japan, and hopefully will be accepted as the first chemopreventive drug for HCC.

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6. REFERENCES

1. Hansen L.A., C.C. Sigman, F. Andreola, S.A. Ross, G.J. Kelloff & L.M. De Luca: Retinoids in chemoprevention and differentiation therapy. *Carcinogenesis* 21, 1271-1279 (2000)
2. Hong W.K. & L.M. Itri: Retinoids and human cancer. In: The reinoids: biology, chemistry, and medicine, 2nd ed. Eds: Sporn M.B., Roberts A.B. & Goodman D.S., Raven Press, New York, 597-630 (1994)
3. Mangelsdorf D.J., K. Umesono & R.M. Evans: The retinoids receptors. In: The reinoids: biology, chemistry, and medicine, 2nd ed. Eds: Sporn M.B., Roberts A.B. & Goodman D.S., Raven Press, New York, 319-349 (1994)
4. Flaumenhaft R., S. Kojima, M. Abe & D.B. Rifkin: Activation of latent transforming growth factor β. In: Advances in pharmacology. Vol 24. Eds: August J.T., Anders M.W. & Murad F., Academic Press, San Diego, 51-76 (1993)
5. Blomhoff R. & K. Wake: Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. *FASEB J* 5, 271-277 (1991)
6. Friedman S.L.: Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 275,2247-2250 (2000)
7. Okuno M., S. Adachi, K. Akita, H. Moriwaki, S. Kojima & S.L. Friedman: Liver fibrosis and hepatic stellate cells. *Connect Tissue* 32, 401-406 (2000)
8. Okuno M., S. Nagase, Y. Shiratori, H. Moriwaki, Y. Muto, N. Kawada & S. Kojima: Retinoid and liver fibrosis. In: Liver diseases and hepatic sinusoidal cells. Eds: Tanikawa K. & Ueno T., Springer-Verlag, Tokyo, 232-241 (1999)
9. Senoo H. & K. Wake: Suppression of experimental hepatic fibrosis by administration of vitamin A. *Lab Invest* 52, 182-194 (1985)
10. Seifert W.F., A. Bosma, A. Brouwer, H. Hendriks, P.J.M. Roholl, R.E.W. van Leeuwen, G.C.F. van Thiel-de Ruiter, I. Seifert-Bock & D.L. Knook: Vitamin A deficiency promotes CCl4 liver fibrosis. *Hepatology* 19, 193-201 (1994)
11. Leo M.A. & C.S. Lieber: Hepatic fibrosis after long-term administration of ethanol and moderate vitamin A supplementation in the rat. *Hepatology* 3, 1-11 (1983)
12. Geubel A.P., C. De Galocsy, N. Alves, J. Rahier & C. Davis: Liver damage caused by therapeutic vitamin A administration: estimate of dose-related toxicity in 41 cases. *Gastroenterology* 100, 1701-1709 (1991)
13. Nicholson R.C., S. Mader, S. Nagpal, M. Leid, C. Rochette-Egly & P. Chambon: Negative regulation of the rat stromelysin gene promoter by retinoic acid is mediated by an AP1 binding site. *EMBO J* 9, 4443-4454 (1991)
14. Pan L., C. Eckhoff & C.E. Brinckerhoff: Suppression of collagenase gene expression by all-*trans* and 9-*cis* retinoic acid is ligand dependent and requires both RARs and RXRs. *J Cell Biochem* 57, 575-589 (1995)
15. Okuno M., H. Moriwaki, S. Imai, Y. Muto, N. Kawada, Y. Suzuki & S. Kojima: Retinoids exacerbate rat liver fibrosis by inducing the activation of latent TGF-β in liver stellate cells. *Hepatology* 26, 913-921 (1997)
16. Border W.A. & N.A. Noble: Transforming growth factor β in tissue fibrosis. *N Engl J Med* 331, 1286-1292 (1994)
17. Roberts A.B. & M.B. Sporn: The transforming growth factor-β s. In: Peptide growth factors and their receptors I. Handbook of experimental pharmacology. Vol 95/I. Eds: Sporn M.B. & Roberts A.B., Springer-Verlag, Berlin 419-472 (1990)
18. Sato Y., R. Tsuboi, R. Lyons, H. Moses & D.B. Rifkin: Characterization of the activation of latent TGF-β by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. *J Cell Biol* 111, 757-763 (1990)
19. Kojima S., P.C. Harpel & D.B. Rifkin: Lipoprotein (a) inhibits the generation of transforming growth factor β: an endogenous inhibitor of smooth muscle cell migration. *J Cell Biol* 113, 1439-1445 (1991)
20. Gudas L.J., M.B. Sporn & A.B. Roberts: Cellular biology and biochemistry of the retinoids. In: The retinoids. biology, chemistry and medicine. 2nd ed. Eds: Sporn M.B., Roberts A.B. & Goodman D.S, Raven Press, New York 443-520 (1994)
21. Kojima S., K. Nara & D.B. Rifkin: Requirement for transglutaminase in the activation of latent transforming growth factor-β in bovine endothelial cells. *J Cell Biol* 121, 439-448 (1993)
22. Kojima S. & D.B. Rifkin: Mechanism of retinoid-induced activation of latent transforming growth factor-β in bovine endothelial cells. *J Cell Physiol* 155, 323-332 (1993)
23. Okuno M., T. Sato, T. Kitamoto, S. Imai, N. Kawada, Y. Suzuki, H. Yoshimura, H. Moriwaki, K. Onuki, S. Masushige, Y. Muto, S.L. Friedman, S. Kato & S. Kojima: Increased 9,13-di-*cis*-retinoic acid in rat hepatic fibrosis: implication for a potential link between retinoid loss and TGF-β mediated fibrogenesis *in vivo*. *J Hepatol* 30, 1073-1080 (1999)
24. Horst R.L., T.A. Reinhardt, J.P. Goff, B.J. Nonnecke, U.K. Gambhir, P.D. Fiorella & J.L. Napoli: Identufication of 9-*cis*, 13-*cis*-retinoic acid as a major circulating retinoid in plasma. *Biochemistry* 34, 1203-1209 (1995)
25. Kojima R., T. Fujimori, N. Kiyota, Y. Toriya, T. Fukuda, T. Ohashi, T. Sato, Y. Yoshizawa, K. Takeyama, H. Mano, S. Masushige & S. Kato: *In vivo* isomerization of retinoic acids. Rapid isomer exchange and gene expression. *J Biol Chem* 269, 32700-32707 (1994)
26. Arnhold T., G. Tzimas, W. Wittfoht, S. Plonait & H. Nau: Identification of 9-*cis*-retinoic acid, 9,13-di-*cis*-rerinoic acid, and 14-hydroxy-4,14-retro-retinol in human plasma after liver consumption. *Life Sci* 59, PL169-177 (1996)
27. Imai S., M. Okuno, H. Moriwaki, Y. Muto, K. Murakami, K. Shudo, Y. Suzuki & S. Kojima: 9,13-di-*cis*-Retinoic acid induces the production of tPA and activation of latent TGF-β via RARα in a human liver stellate cell line, LI90. *FEBS Lett* 411, 102-106 (1997)
28. Ohata M., M. Lin, M. Satre & H. Tsukamoto: Diminished retinoic acid signaling in hepatic stellate cells in cholestatic liver fibrosis. *Am J Physiol* 272, G589-G596 (1997)
29. Okuno M., Y. Muto, H. Moriwaki, M. Kato, M.

Ninomiya, S. Nishiwaki, A. Noma, O. Tagaya, Y. Nozaki & Y. Suzuki: Inhibitory effect of acyclic retinoid (polyprenoic acid) in hepatic fibrosis in CCl4-treated rats. *J Gastroenterol* 25, 223-229 (1990)

30. Inuzuka S., T. Ueno, T. Torimura & K. Tanikawa: The significance of colocalization of plasminogen activator inhibitor-1 and vitronectin in hepatic fibrosis. *Scand J Gastroenterol* 32, 1052-1060 (1997)

31. Gressner A.M. & M.G. Bachem: Molecular mechanisms of liver fibrogenesis – a homege to the role of activated fat-storing cells. *Digestion* 56, 335-346 (1995)

32. Okuno M., H. Moriwaki, Y. Muto & S. Kojima: Protease inhibitors suppress TGF-β generation by hepatic stellate cells. *J Hepatol* 29, 1031-1032 (1998)

33. Sugiyama M., Y. Atomi, N. Wada, A. Kuroda & T. Muto: Effect of oral protease inhibitor administration on gallbladder motility in patients with mild chronic pancreatitis. *J Gastroenterol* 32, 374-379 (1997)

34. Kobayashi I., S. Ohwada, T. Ohya, T. Yokomori, H. Iesato & Y. Morishita: Jejunal pouch with nerve preservation and interposition after total gastrectomy. *Hepato-Gastroenterol* 45, 558-562 (1998)

35. Okuno M., K. Akita, H. Moriwaki, N. Kawada, K. Ikeda, K. Kaneda, Y. Suzuki & S. Kojima: Prevention of rat hepatic fibrosis by the protease inhibitor, camostat mesilate, via reduced generation of active TGF-β. *Gastroenterology* 120,1784-1800 (2001)

36. Leyland H., J. Gentry, M.J. Arthur & R.C. Benyon: The plasminogen-activating system in hepatic stellate cells. *Hepatology* 24, 1172-1178 (1996)

37. Benyon R.C., C.J. Hovell, M. Da Gaca, E.H. Jones, J.P. Iredale & M.J. Arthur: Progelatinase A is produced and activated by rat hepatic stellate cells and promotes their proliferation. *Hepatology* 30, 977-986 (1999)

38. Seki T., H. Imai, S. Uno, T. Ariga & T.D. Gelehrter: Production of tissue-type plasminogen activator (t-PA) and type-1 plasminogen activator inhibitor (PAI-1) in mildly cirrhotic rat liver. *Thromb Haemost* 75, 801-807 (1996)

39. Zhang L.P., T. Takahara, Y. Yata, K. Furui, B. Jin, N. Kawada & A. Watanabe: Increased expression of plasminogen activator and plasminogen activator inhibitor during liver fibrogenesis of rats: role of stellate cells. *J Hepatol* 31, 703-711 (1999)

40. Strobel D., Ch. Wittekind, E. Rouslahti & E.G. Hahn: *In vivo* application of a neutralizing TGF-beta antibody in experimental liver fibrosis. *J Hepatol* 18 (Suppl 1), S63 (1993)

41. Ueno H., T. Sakamoto, T. Nakamura, Z. Qi, N. Atsuchi, A. Takeshita, K. Shimizu & H. Ohashi: A solble transforming growth factor beta receptor expressed in muscle prevents liver fibrogenesis and dysfunction in rats. *Hum Gene Ther* 11, 33-42 (2000)

42. George J., D. Roulot, V.E. Koteliansky & D.M. Bissel: *In vivo* inhibition of rat stellate cell activation by soluble transforming growth factor β type II receptor: a potential new therapy for hepatic fibrosis. *Proc Natl Acad Sci USA* 96, 12719-12724 (1999)

43. Koda H., M. Okuno, S. Imai, H. Moriwaki, Y. Muto, N. Kawada & S. Kojima: Retinoic acid-stimulated liver stellate cells suppress the production of albumin from parenchymal cells via TGF-β. *Biochem Biophys Res Commun* 221, 565-569 (1996)

44. Primary liver cancer in Japan. Eds: Tobe T., Kameda H., Okudaira M., Ohto M., Endo Y., Mito M., Okamoto E., Tanikawa K. & Kojiro M., Springer-Verlag, Tokyo (1992)

45. El-Sarag H.B. & A.C. Manson: Rising incidence of hepatocellular carcinoma in the United States. *N Engl J Med* 340, 745-750 (1999)

46. El-Sarag H.B. & A.C. Manson: Risk factors for the rising rates of primary liver cancer in the United States. *Arch Intern Med* 160, 3227-3230 (2000)

47. Taylor-Robinson S.D., G.R. Foster, S. Arora, S. Hargreaves & H.C. Thomas: Increase in primary liver cancer in the UK, 1979-94. *Lancet* 350, 1142-1143 (1997)

48. Deuffic S., T. Poynard, L. Buffat & A.-J. Valleron: Trends in primary liver cancer. *Lancet* 351, 214-215 (1998)

49. Shiratori Y., H. Yoshida & M. Omata: Different clinicophathological features of hepatocellular carcinoma in relation to causative agents. *J Gastroenterol* 36, 73-78 (2001)

50. Ikeda K., H. Saitoh, I. Koida, Y. Arase, A. Tsubota, K. Chayama, H. Kumada & M. Kawanishi: A multivariate analysis of risk factors of hepatocellular carcinogenesis: A prospective observation of 795 patients with viral and alcoholic cirrhosis. *Hepatology* 18, 47-53 (1993)

51. Adachi E., T. Maeda, T. Matsumura, K. Shirabe, N. Kinukawa, K. Sugimachi & M. Tsuneyoshi: Risk factors for intrahepatic recurrence in human small hepatocellular carcinoma. *Gastroenterology* 108, 768-775 (1995)

52. Kumada T., S. Nakano, I. Takeda, K. Sugiyama, T. Osada, S. Kiriyama, Y. Sone, H. Toyoda, S. Shimada, M. Takahashi & T. Sassa: Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. *Hepatology* 25, 87-92 (1997)

53. Koda M., Y. Murawaki, A. Mitsuda, K. Ohyama, Y. Horie, T. Suou, H. Kawasaki & S. Ikawa: Predictive factors for intrahepatic recurrence after percutaneous ethanol injection therapy for small hepatocellular carcinoma. *Cancer* 88, 529-537 (2000)

54. Okada S., K. Shimada, J. Yamamoto, T. Takayama, T. Kosuge, S. Yamasaki, M. Sakamoto & S. Hirohashi: Predictive factors for postoperative recurrence of hepatocellular carcinoma. *Gastroenterology* 106, 1618-1624 (1994)

55. Chen Y.-J., S.-W. Yeh, J.-T. Chen, C.-C. Wu, M.-T. Hsu, S.-F. Tsai, P.-J. Chen & C.-H. Lin: Choromosomal changes and clonality relationship between primary and recurrent hepatocellular carcinoma. *Gastroenterology* 119, 431-440 (2000)

56. Liver Cancer Study Group of Japan. Survey and follow-up study of primary liver cancer in Japan. *Acta Hepatol Jpn* 38, 317-330 (1997) (in Japanese with English abstract)

57. Chang M.-H., C.-J. Chen, M.-S. Lai, H.-M. Hsu, T.-C. Wu, M.-S. Kong, D.-C. Liang, W.-Y. Shau & D.-S. Chen for the Taiwan Childhood Hepatoma Study Group: Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. *N Engl J Med* 336, 1855-1859 (1997)

58. Nishiguchi S., T. Kuroki, S. Nakatani, H. Horimoto, T. Takeda, S. Nakajima, S. Shiomi, S. Seki, K. Kobayashi & T. Tango: Randomized trial of effects of interferon-alpha on the incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. *Lancet* 346, 1051-1055 (1995)

59. International interferon-alfa HCC study group: Effect of interferon-alfa on progression of cirrhosis to hepatocellular carcinoma: a retrospective cohort study. *Lancet* 351, 1535-1359 (1998)
60. Kasahara A., N. Hayashi, K. Mochizuki, M. Takayanagi, K. Yoshioka, S. Kakumu, A. Iijima, A. Urushihara, K. Kiyosawa, M. Okuda, K. Hino, K. Okita & Osaka Liver Disease Study Group: Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. *Hepatology* 27, 1394-402 (1998)
61. Yoshida H., Y. Shiratori, M. Moriyama, Y. Arakawa, T. Ide, M. Sata, O. Inoue, M. Yano, M. Tanaka, S. Fujiyama, S. Nishiguchi, T. Kuroki, F. Imazeki, O. Yokosuka, S. Kinoyama, G. Yamada & M. Omata for the IHIT Study Group: Interferon therapy reduces the risk for hepatocellular carcinoma: National surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. *Ann Intern Med* 131, 174-181 (1999)
62. Oka H., S. Yamamoto, T. Kuroki, S. Harihara, T. Marumo, S.R. Kim, T. Monna, K. Kobayashi & T. Tango: Prospective study of chemoprevention of hepatocellular carcinoma with Sho-saiko-to (TJ-9). *Cancer* 76, 743-749 (1995)
63. Arase Y., K. Ikeda, N. Murashima, K. Chayama, A. Tsubota, I. Koida, Y. Suzuki, S. Saitoh, M. Kobayashi & H. Kumada: The long term efficacy of glycyrrhizin in chronic hepatitis C patients. *Cancer* 79, 1494-1500 (1997)
64. Muto Y., H. Moriwaki, M. Ninomiya, S. Adachi, A. Saito, K.T. Takasaki, T. Tanaka, K. Tsurumi, M. Okuno, E. Tomita, T. Nakamura & T. Kojima for the Hepatoma Prevention Study Group: Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. *N Engl J Med* 334:1561-1567 (1996)
65. Muto Y., H. Moriwaki, A. Saito: Prevention of second primary tumors by an acyclic retinoid in patients with hepatocellular carcinoma. *N Engl J Med* 340, 1046-1047 (1999)
66. Okuno M., S. Kojima & H. Moriwaki: Chemoprevention of hepatocellular carcinoma: concept, progress and perspectives. *J Gastroenterol Hepatol* 16 (in press) (2001)
67. Gutterman J.U.: Cytokine therapeutics: lessons from interferon alpha. *Proc Natl Acad Sci USA* 91, 1198-1205 (1994)
68. Shiratori Y., N. Kato, O. Yokosuka, F. Imazeki, E. Hashimoto, N. Hayashi, A. Nakamura, M. Asada. H. Kuroda, N. Tanaka, Y. Arakawa & M. Omata for the Tokyo-Chiba hepatitis research group: Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Gastroenterology 113, 558-566 (1997)
69. Lau W.Y., T.W.T. Leung, S.K.W. Ho, M. Chan, D. Machin, J. Lau, A.T.C. Chan, W. Yeo, T.S.K. Mok, S.C.H. Yu, N.W.Y. Leung & P.J. Johnson: Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomized trial. Lancet 353, 797-801 (1999)
70. Takayama T., T. Sekine, M. Makuuchi, S. Yamasaki, T. Kosuge, J. Yamamoto, K. Shimada, M. Sakamoto, S. Hirohashi, Y. Ohashi & T. Kakizoe: Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomized trial. *Lancet* 356, 802-807 (2000)
71. Muto Y. & H. Moriwaki: Antitumor activities of vitamin A and its derivatives. *J Natl Cancer Inst* 73, 1389-1393 (1984)
72. Moriwaki H., Y. Muto, M. Ninomiya, K. Kawai, Y. Suzuki & T. Seto: Inhibitory effects of synthetic acidic retinoid and polyprenoic acid on the development of hepatoma in rats induced by 3'-methyl-N,N-dimethyl-4-aminoazobenzene. *J Gastroenterol* 23, 546-552 (1988)
73. Adachi S., H. Moriwaki, Y. Muto, Y. Yamada, Y. Fukutomi, M. Shimazaki, M. Okuno & M. Ninomiya: Reduced retinoid content in hepatocellular carcinoma with special reference to alcohol consumption. *Hepatology* 14, 776-780 (1991)
74. Garcia A.D., P. Ostapchuk & P. Hearing: Functional interaction of nuclear factors EF-C, HNF-4, and RXRα with hepatitis B virus enhancer 1. *J Virol* 67, 3940-3950 (1993)
75. Matsushima-Nishiwaki R., M. Okuno, S. Adachi, T. Sano, K. Akita, H. Moriwaki, S.L. Friedman & S. Kojima: Phosphorylation of retinoid X receptor α at serine 260 impairs its metabolism and function in human hepatocellular carcinoma. *Cancer Res* 61, 7675-7682 (2001)
76. Kakizuka A., W.H.Jr. Miller, K. Umesono, R.P.Jr. Warrell, S.R. Frankel, V.V.V.S. Murty, E. Dmitrovsky & R.M. Evans: Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR-α with a novel putative transcription factor, PML. *Cell* 66, 663-674 (1991)
77. Nakamura N., Y. Shidoji, Y. Yamada, H. Hatakeyama, H. Moriwaki & Y. Muto: Induction of apoptosis by acyclic retinoid in the human hepatoma-derived cell line, HuH-7. *Biochem Biophys Res Commun* 207, 382-388 (1995)
78. Moriwaki H., I. Yasuda, Y. Shiratori, T. Uematsu, M. Okuno & Y. Muto: Deletion of serum lectin-reactive α-fetoprotein by acyclic retinoid: a potent biomarker in the chemoprevention of second primary hepatoma. *Clin Cancer Res* 3, 727-731 (1997)
79. Shiraki K., K. Takase, Y. Tameda, M. Hamada, Y. Kosaka & T. Nakano: A clinical study of lectin-reactive α-fetoprotein as an early indicator of hepatocellular carcinoma in the follow-up of cirrhotic patients. *Hepatology* 22, 802-807 (1995)
80. Moriwaki H., M. Okuno, Y. Shiratori, I. Yasuda & Y. Muto: Clonal deletion, a novel strategy of cancer control that falls between cancer chemoprevention and cancer chemotherapy: a clinical experience in liver cancer. In: Frontiers in hepatology. Progress in hepatocellular carcinoma treatment. Ed: Okita K., Springer-Verlag, Tokyo, 97-103 (2000)
81. Okuno M., T. Sano, R. Matsushima-Nishiwaki, S. Adachi, K. Akita, Y. Okano, S. Kojima & H. Moriwaki: Apoptosis induction by acyclic retinoid: a molecular basis of 'clonal deletion' therapy for hepatocellular carcinoma. *Jpn J Clin Oncol* 31, 358-362 (2001)
82. Huang M.-E., Y. Yu-Chen, C. Shu-Rong, M.-X. Lu, L. Zhao, L.-J. Gu & Z.-Y. Wang: Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 72, 567-572 (1988)
83. Yamada Y., Y. Shidoji, Y. Fukutomi, T. Ishikawa, T. Kaneko, H. Nakagama, M. Imawari, H. Moriwaki & Y. Muto: Positive and negative regulations of albumin gene

expression by retinoids in human hepatoma cell lines. *Mol Carcinog* 10, 151-158 (1994)
84. Nagy L., V. A. Thomazy, R.A. Heyman & P.J.A. Davies: Retinoid-induced apoptosis in normal and neoplastic tissues. *Cell Death Different* 5, 11-19 (1998)
85. Hong W.K., S.M. Lippman, W.N. Hittelman & R. Lotan: Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. *Clin Cancer Res* 1, 677-686 (1995)
86. Lotan R.: Retinoids in cancer in cancer chemoprevention. *FASEB J* 10, 1031-1039 (1996)
87. Lotan R.: Retinoids and apoptosis: implications for cancer chemoprevention and therapy. *J Natl Cancer Inst* 22, 1655-1657 (1995)
88. Ma X., S. Karra, W. Guo, D.J. Lindner, J. Hu, J.E. Angell, E.R. Hofmann, S.P.M. Reddy & D.V. Kalvakolamu: Regulation of interferon and retinoic acid-induced cell death activation through thioredoxin reductase. *J Biol Chem* 276, 24843-24854 (2001)

Abbreviations: HCC, hepatocellular carcinoma; RA, retinoic acid; RBP, retinol-binding protein; CRBP, cellular retinol-binding protein; CRABP, cellular retinoic acid-binding protein; RAR, retinoic acid receptor; RXR, retinoid X receptor; atRA, all-*trans* RA; 9cRA 9-*cis* RA; RARE, RA response element; RXRE, RXR response element; HPC, hepatic parenchymal cell; HSC, hepatic stellate cell; ECM, extracellular matrix; TGF-β, transforming growth factor-β; PA, plasminogen activator; FGF, fibroblast growth factor; αSMA, α-smooth muscle actin; PAI-1, PA inhibitor-1; LPS, lipopolysaccharide; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; MAP kinase, mitogen-activated protein kinase; APL, acute promyelocyte leukemia; 3'MeDAB, 3'-methyl-4-dimethylaminoazobenzene; CCC, cholangiocellular carcinoma; DEN, dimethylnitrosamine; AFP-L3, lectin-reactive α-fetoprotein fraction 3; ADH, alcohol dehydrogenase; AHD, aldehyde dehydrogenase; HPLC, high performance liquid chromatography; uPA, urokinase type PA.

Key Words: Hepatocellular Carcinoma, Retinoic Acid, Retinol-Binding Protein, Cellular Retinol-Binding Protein, Cellular Retinoic Acid-Binding Protein, Retinoic Acid Receptor, Retinoid X Receptor, All-Trans, Ra Response Element, Response Element, Hepatic Parenchymal Cell, Hepatic Stellate Cell, Extracellular Matrix, Transforming Growth Factor-Beta, Plasminogen Activator, Fibroblast Growth Factor, Smooth Muscle Actin, Pa Inhibitor-1, Lipopolysaccharide, Hepatitis B Virus, Hepatitis C Virus, Interferon, Mitogen-Activated Protein Kinase, Acute Promyelocyte Leukemia, Cholangiocellular Carcinoma, Dimethylnitrosamine, Lectin-Reactive alpha-Fetoprotein Fraction, Alcohol Dehydrogenase, Aldehyde Dehydrogenase, High Performance Liquid Chromatography, Urokinase, Review

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