Table 2 Guidelines for the treatment of patients with chronic hepatitis B aged 35 years or older

Eligibility criteria	ALT	≥31 IU/L
	HBV DNA	HBeAg-positive patients: ≥5 log copies/mL HBeAg-negative patients: ≥4 log copies/mL Patients with cirrhosis: ≥3 log copies/mL

HBV DNA	≥7 log copies/mL	<7 log copies/mL
HBeAg-positive	(1) Entecavir (2) Sequential treatment† (entecavir plus IFN)	(1) Entecavir (2) Long-term IFN for 24–48 weeks
HBeAg-negative	Entecavir	(1) Entecavir (2) Long-term IFN for 24–48 weeks

†Sequential treatment: patients who have lost hepatitis B virus (HBV) DNA after treatment with nucleot(s)ide analog receive combined interferon (IFN) for 4 weeks, and then IFN monotherapy is continued for 20 weeks, and lifted thereafter. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.

accordingly, they would be resistant to interferon (IFN) therapy. Should they receive nucleos(t)ide analogs, however, the duration would become inevitably longer, because they start the treatment when younger than 35 years old. Hence, IFN for 24–48 weeks is the first choice in their treatment. The standard treatment of 3 months is favored, which can be extended to the maximum of 6 months. Non-pegylated (standard) IFN-α is recommended to them, because self-injection at home is approved for preparations of IFN-α; it helps improve their quality of life (QOL). There are many patients who are refractory to IFN and in whom improvement of ALT levels and/or decrease in HBV DNA titers are hardly achievable. Therefore, as another option, monotherapy with entecavir can be applied for the purpose of clearing HBeAg from serum and lowering HBV DNA titers. For HBeAg-positive patients with lower HBV DNA titers (<7 log copies/mL), also, long-term IFN is endorsed as a rule.

There are HBeAg-negative patients in whom ALT levels increase to 31 IU/mL or more repeatedly. In the 2008 guidelines, sequential treatment with IFN and entecavir is introduced as a new arm of therapeutic options for such patients.[1]

For HBeAg-negative patients with less than 7 copies/mL of HBV DNA, in general, regular follow up without therapeutic intervention is deemed to suffice for the majority. For those of them in whom ALT levels flare to 31 IU/mL or more time after time, long-term IFN for 24 weeks is indicated. Because liver disease progresses in many HBeAg-negative patients, for those with platelet counts of less than $150 \times 10^3/mm^3$ or in fibrosis stage F2 or higher, treatment with entecavir is indicated.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B AGED 35 YEARS OR OLDER

TABLE 2 SUMS up treatment modalities for patients with chronic hepatitis B who are aged 35 years or older. HBeAg-positive patients in this age range who carry HBV DNA in titers of 7 log copies/mL or more rarely, if ever, seroconvert to the loss of HBeAg by IFN-based therapies. Hence, entecavir is the first choice in their treatment.[2,3] Because HBV mutants resistant to entecavir can be elicited by it, sequential treatment with IFN plus entecavir is amended in the 2008 guidelines.[1] In view of low viral loads in patients who possess HBV DNA in titers of less than 7 log copies/mL, entecavir is selected as the first choice, followed by long-term IFN as the second choice of treatment in these patients. HBeAg-negative patients who have high viral loads (≥7 log copies/mL), on the other hand, can normalize ALT levels by monotherapy with entecavir. Therefore, entecavir becomes their first choice, and this is the case even in patients with HBV DNA titers less than 7 copies/mL.

GUIDELINES FOR THE TREATMENT WITH NUCLEOS(T)IDE ANALOGS OF PATIENTS WITH CHRONIC HEPATITIS B WHO ARE RECEIVING LAMIVUDINE

TABLE 3 DETAILS guidelines for the treatment with nucleos(t)ide analogs of patients with chronic hepatitis B who are receiving lamivudine. Because a number of drug-resistant HBV mutants emerge increasingly with time in patients on long-term treatment with lamivudine, the fundamental rule is to switch them to ente-

Table 3 Guidelines for the treatment with nucleos(t)ide analogs in patients with chronic hepatitis who are receiving lamivudine

Lamivudine	Less than 3 years		3 years or longer
HBV DNA			
<1.8 log copies/mL persistently	May be switched to entecavir 0.5 mg daily		Continued on lamivudine 100 mg daily
≥1.8 log copies/mL	VBT (−)	May be switched to entecavir 0.5 mg daily	
	VBT (+)	Adefovir 10 mg daily add-on lamivudine	Adefovir 10 mg daily add-on lamivudine

HBV, hepatitis B virus; VBT, virological breakthrough.

cavir. For this reason, patients are stratified by the duration of lamivudine treatment, less than 3 years and 3 years or more, as well as HBV DNA titers persistently below 1.8 log copies/mL and 1.8 log copies/mL or more, and separate treatment strategies have been worked out for the patients in each category. Because by far the majority of patients with a duration of lamivudine treatment of less than 3 years and HBV DNA titers of less than 1.8 copies/mL possess drug-resistant mutants in low frequencies, they are recommended to switch to entecavir 0.5 mg daily as soon as possible. Likewise, patients who have received lamivudine for 3 years or longer, but in whom drug-resistant mutants have never developed, are recommended to switch to entecavir 0.5 mg daily. By contrast, for patients in whom drug-resistant mutants have emerged already and who have undergone virological breakthroughs,[4] adefovir 10 mg daily add-on lamivudine is started for the purpose of stabilizing liver function.[5] In regard of the patients who have received lamivudine for 3 years or longer, those without drug-resistant mutants can stay on lamivudine 100 mg daily.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS B (PART I)

FOR THE FISCAL year 2008, the following three items have been added to previous guidelines for the treatment of chronic hepatitis B (Table 4).

1 In the treatment of patients with chronic hepatitis B, IFN is the first resort for those younger than 35 years, toward the eventual goal of gaining a "drug-free state". For the patients aged 35 years or older, persistently negative HBV DNA is the aim of nucleos(t)ide analogs, with the first choice being entecavir in their primary treatment. On the other hand, for patients with HBV mutants resistant to lamivudine and/or entecavir, combined treatment with adefovir and lamivudine is the principal rule (Table 3).[6–8]
2 Therapeutic responses to antiviral treatment are much different in patients with chronic hepatitis B who are infected with HBV of distinct genotypes. It is recommended therefore to determine HBV genotypes before making a decision on the treatment choice. In particular, the patients infected with HBV of genotype A or B respond to IFN in high rates, even if they are aged 35 years or older. For these reasons, IFN becomes the first choice in their antiviral treatment.
3 The duration of IFN treatment is 24 weeks basically. In the patients in whom the efficacy of IFN has been achieved with decrease in HBV DNA titers and normalization of ALT, the treatment duration is better extended to 48 weeks.

Table 4 Supplements to guidelines for the treatment of patients with chronic hepatitis B (part I)

1 Treatment of patients with chronic hepatitis B aims at a "drug-free state" by IFN-based therapies in those younger than 35 years, and at persistently negative HBV DNA in those aged 35 years or older, with entecavir as the first choice in the primary therapy. Lamivudine plus adefovir forms the basis for the treatment of HBV mutants resistant to lamivudine or entecavir.
2 In view of antiviral response much different in patients infected with HBV of distinct genotypes, it is desired to make treatment choices based on genotypes. In particular, because genotypes A and B respond to IFN with high efficacy, even in patients aged 35 years or older, IFN is recommended as the first treatment choice in these patients.
3 The duration of IFN is for 24 weeks basically, but extension to 48 weeks is recommended in patients who respond to IFN with decrease in HBV DNA titers and normalization of ALT levels.

ALT, alanine aminotransferase; HBV, hepatitis B virus; IFN, interferon.

Table 5 Supplements to guidelines for the treatment of patients with chronic hepatitis B (part II)

- Self-injection of IFN at home is recommended to patients, who are eligible to do it, for improving their quality of life.
- Treatment with nucleos(t)ide analogs should be continued in patients in whom cirrhosis or HCC has been cured.
- Antiviral treatment is considered in patients with ALT levels of ≥31 IU/L. To patients aged 35 years or older in whom viral replication persists, even to those with normal ALT levels, antiviral treatments are indicated. It is possible, however, to follow for outcomes in patients who are elderly or HBeAg-negative and in whom antiviral treatments are difficult, while they receive liver supportive therapy (e.g. SNMC, UDCA).
- In patients co-infected with HBV and HIV, entecavir cannot be used due to the possibility for emergence of HIV variants resistant to antiretroviral therapies.
- Immunosuppressive and anticancer drugs should be used with utmost caution, even in patients with low HBV DNA titers and normal ALT levels, because they can induce severe liver damage along with elevation in HBV DNA titers.

ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; IFN, interferon; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS B (PART II)

FURTHER, THE FOLLOWING five supplements have been added to the 2008 guidelines (Table 5).

To patients who are eligible, self-injection of IFN at home is recommended, taking into consideration their QOL. Because IFN-based therapies are not recommended for patients in whom HBV has been transmitted by perinatal infection, sequential treatment with IFN plus entecavir serves as another option in their antiviral treatment.

Treatment with nucleos(t)ide analogs should be extended to patients in whom cirrhosis or hepatocellular carcinoma (HCC) has been cured after successful therapies.

Antiviral treatment has to be considered in patients with ALT levels of 31 IU/L or more. Patients aged 35 years or older with normal ALT levels but in whom HBV replication persists, need to be considered for antiviral treatments. Elderly and HBeAg-negative patients, as well as those to whom the administration of antiviral drugs is difficult, can be followed regularly while they receive liver supportive therapy (e.g. stronger neo-minophagen C,[9] ursodeoxycholic acid [UDCA][10]).

Patients co-infected with HBV and HIV type 1 cannot receive entecavir due to the possibility of emergence of HIV mutants resistant to antiretroviral drugs.

Even in patients with low HBV DNA titers and normal ALT levels, HBV DNA loads can increase massively to induce severe liver damages in them, while they receive immunosuppressive or anticancer drugs. Hence, utmost caution should be exercised if they are to undergo antiviral treatments.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CIRRHOSIS DUE TO HBV

TABLE 6 SUMMARIZES guidelines for the treatment of patients with type B cirrhosis. Patients with compensated or decompensated cirrhosis, who are infected with HBV, receive entecavir for persistent clearance of HBV DNA detectable by the real-time polymerase chain reaction and normalization of aspartate aminotransferase as well as ALT levels. Combined lamivudine plus adefovir therapy are indicated for patients in whom HBV mutants resistant to lamivudine or entecavir have developed. Guidelines for maintaining liver function, for preventing the development of HCC, include liver supportive therapy with glycyrrhizin and UDCA, either alone or in combination. For treatment toward sup-

Table 6 Guidelines for treatment of type B cirrhosis

Principles

Compensated: termination of HBV infection by antiviral treatment with entecavir as the mainstay.

Decompensated: reversal to compensation and prevention of HCC.

Methods

(1) Eradication of HBV and normalization of ALT/AST (compensated and decompensated cirrhosis).
   a) Entecavir.
   b) Combined lamivudine and adefovir (for patients with HBV mutants resistant to lamivudine or entecavir).

(2) Maintenance of liver function (improvement of ALT/AST and albumin) for preventing HCC.
   a) Liver supportive therapy such as SNMC or UDCA.
   b) Branched chain amino acids (Livact).

(3) Supplementation with nutrients (for stabilizing liver function in decompensated cirrhosis).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

pressing the development of HCC, branched chain amino acids (BCAA)[11] are implemented. Also, nutrient supplements are utilized for stabilizing liver function.

DISCUSSION AND CONCLUSION

THE STUDY GROUP for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, organized by the Ministry of Health, Labor and Welfare of Japan, has compiled a series of guidelines for the treatment of liver disease due to HBV and HCV ranging from chronic hepatitis to cirrhosis of various severities annually, since the fiscal year 2002. The principal aim of these guidelines is to decrease the incidence of HCC due to hepatitis virus infections in Japan. In accordance with this principle, supplements have been added to previous guidelines for the standardization of treatment of chronic viral liver disease every fiscal year. This article summarizes guidelines for the treatment of liver disease due to HBV. Guidelines for the treatment of liver disease due to HCV for the fiscal year 2008 are reported in the accompanying paper. They are formulated on evidence-based data that have been accumulated by members and cooperators of the study group. It will be necessary to improve these guidelines in the next fiscal year and henceforth, in accordance with many pieces of new evidence that are expected to evolve through enduring efforts and keen insights of members and cooperators of the study group.

In the treatment of chronic hepatitis B, novel therapeutic strategies have continued to evolve in previous guidelines. In guidelines of the fiscal year 2008, diverse new treatment arms are introduced for gaining the eventual goal of the "drug-fee state".

The Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis has been drafted and displayed on the web site (www.jsh.or.jp/medical/index.html [in Japanese]) as well, guidelines for the treatment of a spectrum of liver diseases due to HBV, ranging from chronic hepatitis to cirrhosis of various severities for the fiscal year 2008. In view of the eventual goal of decreasing the incidence of HCC due to HBV infection, supplementation and adjustment are appended to previous guidelines, and new guidelines have been introduced to the treatment of cirrhosis due to HBV infection. As a general rule, antiviral treatments are the mainstay in guidelines for the treatment of chronic hepatitis B. In addition to them, it is necessary to always keep in mind the fundamental concepts of these guidelines. It is our sincere hope that, for the treatment of each patient, readers will conduct their clinical practice on the basis of these concepts, and then refer to appropriate individual guidelines, when they make decisions regarding treatment strategy, on a case-by-case basis. With respect to guidelines for the treatment of patients with cirrhosis, above all, expected achievable outcomes have to be taken into account in making treatment choices.

We can foretell that there is no end to the treatment of patients with chronic hepatitis and cirrhosis due to HBV, as it will keep evolving and improving in future guidelines. The enduring efforts of doctors and scientists, in pursuit of this goal, will fill in wide social and economic gaps in medical practices being served to the nation, and produce substantial and efficient interest in the medical economy on a national basis. In conducting treatment of patients with liver disease due to HBV infection, according to these guidelines, many new and unforeseen facets may surface that will require further improvements. Hence, it will be necessary to evaluate the therapeutic efficacy of these guidelines, and revise or add necessary supplements to them as required in the future.

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Review Article

Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan

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In the 2008 guidelines for the treatment of patients with chronic hepatitis C, pegylated interferon (Peg-IFN) combined with ribavirin for 48 weeks are indicated for treatment-naive patients infected with hepatitis C virus (HCV) of genotype 1. Treatment is continued for an additional 24 weeks (72 weeks total) in the patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA during 13–36 weeks on treatment. Re-treatment is aimed to either eradicate HCV or normalize transaminase levels for preventing the development of hepatocellular carcinoma (HCC). For patients with compensated cirrhosis, the clearance of HCV RNA is aimed toward improving histological damages and decreasing the development of HCC. The recommended therapeutic regimen is the initial daily dose of 6 million international units (MIU) IFN continued for 2–8 weeks that is extended to longer than 48 weeks, if possible. IFN dose is reduced to 3 MIU daily in patients who fail to clear HCV RNA by 12 weeks for preventing the development of HCC. Splenectomy or embolization of the splenic artery is recommended to patients with platelet counts of less than 50 × 103/mm³ prior to the commencement of IFN treatment. When the prevention of HCC is at issue, not only IFN, but also liver supportive therapy such as stronger neo-minophagen C, ursodeoxycholic acid, phlebotomy, branched chain amino acids (BCAA), either alone or in combination, are given. In patients with decompensated cirrhosis, by contrast, reversal to compensation is attempted.

Key words: chronic hepatitis, cirrhosis, hepatocellular carcinoma, hepatitis C virus, interferon, liver supportive therapy, pegylated interferon, ribavirin

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INTRODUCTION

SINCE THE FISCAL year 2002, guidelines for the treatment of patients with viral hepatitis have been compiled annually by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, under the auspice of the Ministry of Health Labor and Welfare of Japan, recruiting many specialists from all over the nation. They have been improved every year with many supplementary issues that have evolved, as our understanding of various aspects of viral hepatitis deepens and treatment options widen with time. For the fiscal year 2008, guidelines have been worked out for a comprehensive standardization of the treatment of chronic hepatitis and cirrhosis due to infection with hepatitis C virus (HCV) in Japan. It is hoped that these guidelines will be accepted widely and implemented for helping as many patients as possible who suffer from sequelae of persistent HCV infection.

Here, we relate excerpts of the 2008 guidelines for the treatment of patients with HCV-induced liver disease covering a wide range from those with normal aminotransferase levels to those with decompensated cirrhosis.

GUIDELINES FOR THE PRIMARY TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C

TABLE 1 SUMMARIZES the antiviral therapy of treatment-naive patients with chronic hepatitis C. In comparison with previous guidelines, the duration of combined treatment with pegylated interferon (Peg-IFN) and ribavirin is extended to 48–72 weeks for patients infected with HCV of genotype 1 in high viral loads (HVL: ≥5 log IU/mL by the Japanese criteria).[1,2] For patients infected with HCV of genotype 2 in HVL, Peg-IFN-α2b and ribavirin for 24 weeks are indicated. To patients with HCV-1 in low viral loads (LVL: <5 log IU/mL), either the standard IFN (not conjugated with polyethylene glycol) for 24 weeks, or the weekly monotherapy with Peg-IFN-α2a for 24–48 weeks, is given.[3] Patients with HCV-2 in LVL receive either the standard IFN for 8–24 weeks, or the weekly monotherapy with Peg-IFN-α2a for 24–48 weeks.

GUIDELINES FOR THE RE-TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C

FOR PATIENTS WHO receive re-treatment, first, it is imperatively prerequisite to: (i) identify factors for non-response to previous treatments; and (ii) decide whether to aim for clearance of HCV or to prevent the progression of hepatitis that can accelerate the development of hepatocellular carcinoma (HCC), and this can be monitored by alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels toward normalizing or stabilizing their levels (Table 2).[4] Second, IFN combined with ribavirin is the mainstay of re-treatment of patients with chronic hepatitis C. Third, long-term IFN monotherapy is recommended to patients who are not indicated to IFN/ribavirin or who have failed to respond to the combination therapy. However, some patients do not tolerate IFN due to side-effects or their complicating morbidities. In addition, IFN monotherapy does not always improve ALT levels. Such patients need to receive liver supportive therapy including stronger neo-minophagen C (SNMC)[5] and ursodeoxycholic acid (UDCA),[6] as well as phlebotomy, either alone or in combination. Therapeutic target ALT levels are: (i) within ×1.5 the upper limit of normal (ULN) for patients in fibrosis stage 1 (F1); and (ii) less than 30 IU/L in those in fibrosis stages 2 or 3 (F2/F3), as far as possible.

Table 1 Guidelines for the primary treatment of patients with chronic hepatitis C

Genotypes	Genotype 1	Genotype 2
Viral loads		
High viral load ≥5.0 log IU/mL ≥300 fmol/L ≥1 Meq/mL	• Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 48–72 weeks • Peg-IFN-α2a (Pegasys) + ribavirin (Copegus) for 48–72 weeks	• Peg-IFN-α2b (Peg-Intron) + ribavirin (Rebetol) for 24 weeks
Low viral load <5.0 log IU/mL <300 fmol/L <1 Meq/mL	• Standard IFN for 24 weeks • Peg-IFN-α2a (Pegasys) for 24–48 weeks	• Standard IFN for 8–24 weeks • Peg-IFN-α2a (Pegasys) for 24–48 weeks

Peg-IFN, pegylated interferon.

Table 2 Guidelines for re-treatment of chronic hepatitis C

Principles

Selection has to be made between termination of HCV infection and normalization/stabilization of ALT as well as AFP levels (toward preventing aggravation of liver disease and development of HCC), after evaluating factors for non-response in the primary IFN treatment.

1 "IFN plus ribavirin" is the mainstay of re-treatment of patients who have failed to respond to the primary IFN therapy.
2 Long-term IFN is recommended to patients in whom ribavirin is not indicated or who have failed to respond to IFN/ribavirin; self-injection at home is approved for IFN-α (not for Peg-IFN).
3 Patients who are not indicated to IFN or have failed to improve ALT and AFP levels, in response to IFN, receive liver supportive therapy (SNMC, UDCA) and phlebotomy, either alone or in combination.
4 For preventing aggravation of liver disease (and development of HCC), ALT levels need to be controlled within 1.5 × ULN in patients in stage 1 fibrosis (F1), and as far as possible, 30 IU/L or lower in those in fibrosis stages 2–3 (F2/F3).
5 In treatment combined with ribavirin, dose and mode need to be selected, taking into consideration factors contributing to the response, such as age, sex, progression of liver disease, mutations in the HCV genome (amino acid substitutions in the core protein [aa70/aa91] and ISDR) and HCV RNA titers determined by the real-time PCR.

AFP, α-fetoprotein; ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; PCR, polymerase chain reaction; Peg-IFN, pegylated interferon; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid; ULN, upper limit of normal.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CHRONIC HEPATITIS C

FOR THE FISCAL year 2008, the following items were supplemented to the treatment of chronic hepatitis C (Table 3).

1 The treatment of patients infected with HCV-1 in HVL with Peg-IFN/ribavirin for 72 weeks is modified by the early virological response (EVR) within 12 weeks after the start. Patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA till 13–36 weeks on treatment.[1,2]
2 Patients with HCV-1 in HVL who fail to clear HCV RNA detectable by real-time PCR but in whom ALT levels normalize are continued on Peg-IFN/ribavirin until 48 weeks, so that normalized ALT levels endure longer after the completion of therapy.[7]
3 Patients who are not indicated to Peg-IFN/ribavirin, or who have failed to respond to previous treatments, receive long-term IFN monotherapy. During the first 2 weeks, IFN in the conventional dose is given daily or three times a week. Patients who do not clear HCV RNA during the maximal treatment period of 8 weeks receive half the conventional dose of IFN indefinitely.[8]

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS C IN NORMAL ALT LEVELS

AS IN PREVIOUS guidelines, patients with chronic hepatitis C having normal ALT levels are stratified into four groups by ALT levels and platelet counts (Table 4). Patients with chronic hepatitis C who have normal ALT levels are reported to gain the sustained virological response (SVR) to antiviral treatments comparably frequently as those having elevated ALT levels. Taking this into consideration, patients with ALT levels of 30 IU/L or less and platelet counts of $150 \times 10^3/mm^3$ or more are followed for ALT every

Table 3 Supplements to guidelines for chronic hepatitis C

1 Criteria for extending the duration of Peg-IFN/ribavirin (to 72 weeks) in patients infected with HCV-1b in HVL: patients who have remained positive for HCV RNA detectable by the real-time polymerase chain reaction at 12 weeks after the start of treatment, but who turn negative for HCV RNA till 13–36 weeks on treatment.[1,2]
2 Patients with HCV-1b in HVL who fail to lose HCV RNA detectable by real-time PCR, but in whom ALT levels normalize by 36 weeks, Peg-IFN/ribavirin is given till 48 weeks for maintaining normalized ALT levels long after the completion of treatment.
3 Long-term IFN monotherapy in patients who are not indicated to Peg-IFN/ribavirin, or have failed to respond to it: the usual dose of IFN daily or three times in week is given for the first 2 weeks, and when HCV RNA does not disappear within the maximal duration of 8 weeks, long-term treatment with half the usual dose of IFN is continued indefinitely.

ALT, alanine aminotransferase; HCV, hepatitis C virus; HVL, high viral loads; PCR, polymerase chain reaction; Peg-IFN, pegylated interferon.

Table 4 Guidelines for the treatment of patients with normal ALT levels toward preventing the development of HCC

Platelets	$\geq 150 \times 10^3/mm^3$	$<150 \times 10^3/mm^3$
ALT		
≤30 IU/L	• Follow for ALT every 2–4 months. • If ALT levels elevate, start antiviral treatments taking into consideration the possibility of SVR and risk for HCC.	• Liver biopsy, if possible, and consider antiviral treatments for patients in A2/F2. • Follow for ALT every 2–4 months, and consider antiviral treatments when ALT levels elevate, for patients without biopsy.
31–40 IU/L	• Consider antiviral treatments for patients younger than 65 years.	• Start treatments for chronic hepatitis C. • Select treatments according to genotypes, viral load, age of patients, etc.

ALT, alanine aminotransferase; HCC, hepatocellular carcinoma; SVR, sustained virological response.

2–4 months. If ALT levels increase in them, antiviral treatments are considered based on the possibility of resolving HCV infection and the risk for developing HCC. In view of significant fibrosis present in patients with platelet counts of less than $150 \times 10^3/mm^3$, they are recommended to receive liver biopsy, if this is possible. Patients in fibrosis stage F2 or higher are evaluated for the indication to antiviral treatments. Patients with ALT levels between 31 and 40 IU/L are classified by platelet counts. Antiviral treatments are considered in those aged younger than 65 years who have platelet counts of $150 \times 10^3/mm^3$ or more, while guidelines for patients with chronic hepatitis are applied to those with platelet counts of less than $150 \times 10^3/mm$.[9,10]

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CIRRHOSIS DUE TO HCV

PATIENTS WITH COMPENSATED cirrhosis who are not infected with HCV-1 in HVL receive either IFN-β or IFN-α (Table 5). Since the fiscal year 2008, IFN-α has been approved for the treatment of patients infected with HCV-1 in HVL, with the aim of resolving infection and normalizing ALT as well as AFP levels by long-term therapy. Treatment duration was set at 1 year or longer, and because the longer the treatment duration the higher the SVR rate, 36 weeks has been recommended as the optimal treatment duration. Because the normalization of ALT/AST is important, even in patients who fail to clear HCV infection by these therapeutic regimens, treatment is better conducted for maintaining normal ALT/AST levels. Guidelines for maintaining liver function for preventing the development of HCC include liver supportive therapy with glycyrrhizin[5] and UDCA,[6] either alone or in combination. For treatment toward suppressing the development of HCC, branched chain amino acids (BCAA)[11] or phlebotomy are adopted. Also, nutrient supplements are applied for stabilizing liver function.

SUPPLEMENTS TO GUIDELINES FOR THE TREATMENT OF CIRRHOSIS DUE TO HCV

THE FOLLOWING ITEMS have been appended to supplement guidelines for the treatment of type C cirrhosis (Table 6).

Table 5 Guidelines for treatment of type C cirrhosis

Principles
Compensated: termination of HCV infection
Decompensated: reversal to compensation and prevention of HCC
Methods
(1) Eradication of HCV and normalization of ALT/AST (for patients with compensated cirrhosis).
a) HCV-1b in HVL (≥5 log IU/mL)
IFN-α (Sumiferon)
b) Others
IFN-α (Sumiferon)
IFN-β (Feron)
(2) Maintenance of liver function (improvement of ALT/AST and albumin) for preventing HCC.
a) Liver supportive therapy
Stronger neo-minophagen C (SNMC), ursodeoxycholic acid (UDCA), etc.
b) Branched chain amino acids (BCAA [Livact])
c) Phlebotomy
(3) Supplementation with nutrients (for stabilizing liver function in decompensated cirrhosis).

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVL, high viral loads; IFN, interferon.

Table 6 Supplements to guidelines for type C cirrhosis

1	To start with, IFN for compensated cirrhosis is desired at 6 MIU daily for 2–8 weeks, as far as possible, and to continue for 48 weeks or longer, as for chronic hepatitis C.
2	In patients with compensated cirrhosis who fail to clear HCV RNA within 12 weeks on IFN, long-term therapy at 3 MIU should be considered for preventing HCC.
3	In patients with platelet counts $<50 \times 10^3/mm^3$, splenectomy or embolization of splenic artery is recommended before re-treatment, and after thorough evaluation has been made on the response to IFN to be expected.
4	For the prevention of HCC, not only IFN, but also liver supportive therapy (SNMC, UDCA, etc.), phlebotomy and branched chain amino acids, either alone or in combination, are recommended for improving ALT/AST and AFP levels.

AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; MIU, million international units; SNMC, stronger neo-minophagen C; UDCA, ursodeoxycholic acid.

1 For treatment of type C cirrhosis with IFN, the initial dose of 6 million international units (MIU) daily is continued as long as possible (2–8 weeks). Thereafter, long-term IFN for 48 weeks or longer is desired as in the treatment of chronic hepatitis C.
2 In the treatment of type C cirrhosis, patients who fail to achieve EVR with the clearance of HCV RNA from serum within 12 weeks should receive long-term IFN at a dose of 3 MIU.
3 For patients with type C cirrhosis who have platelet counts of less than $50 \times 10^3/mm^3$, splenectomy or embolization of the splenic artery is desirable before commencing IFN therapy, after the efficacy of IFN has been evaluated thoroughly.[12]
4 For preventing the development of HCC, improvement in ALT, AST and AFP levels are aimed. Toward this end, not only IFN, but also liver supportive therapy (SNMC and UDCA), phlebotomy and BCAA are used, either alone or in combination.

DISCUSSION AND CONCLUSION

THE STUDY GROUP for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, organized by the Ministry of Health, Labor and Welfare of Japan, has compiled a series of guidelines for the treatment of liver disease due to HCV ranging from chronic hepatitis to cirrhosis of various severities for the fiscal year 2008. The principal aim of these guidelines is to decrease the incidence of HCC due to HCV infection in Japan. In accord with this principle, supplements have been added to previous guidelines for the standardization of treatment of chronic hepatitis C. They are prepared on evidence-based data that have been accumulated by members and cooperators of the study group. It is necessary to improve these guidelines in the next fiscal year and thereafter, in accordance with many pieces of new evidence that are expected to emerge through enduring efforts of members and cooperators of the study group.

In the treatment of chronic hepatitis C, the duration of antiviral treatments is extended to 72 weeks, which has been approved as of the fiscal year 2008, and criteria for the eligibility of extended treatment duration are clearly defined. Long-term antiviral treatments, extended up to 72 weeks, are hoped to increase the SVR even further. In addition, comprehensive guidelines for the treatment of cirrhosis have been improved with substantial additions, and their criteria for the indication made explicit.

The Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis has drafted, and also displayed online (www.jsh.or.jp/medical/index.html [in Japanese]), guidelines for a spectrum of liver diseases due to HCV, from chronic hepatitis to cirrhosis of various severities. In view of the eventual goal of decreasing the incidence of HCC due to HCV infection, supplementation and adjustment are appended to previous guidelines, and new guidelines have been constructed for the treatment of cirrhosis due to HCV infection. As a general rule, antiviral treatments constitute the main body of guidelines for the treatment of chronic hepatitis C. Furthermore, the fundamental concept of these guidelines would need to be kept in mind always. It is our sincere hope that, for the treatment of each patient, readers will base their clinical practice on these guidelines, and refer to appropriate individual guidelines, when they make a decision on the treatment strategy, on a case-by-case basis. With respect to guidelines for the treatment of patients with cirrhosis, above all, expected achievable outcomes have to be taken into account in treatment choice.

It is our sincere desire that treatment of patients with chronic hepatitis and cirrhosis due to HCV will proceed following these guidelines. Efforts along these lines will rectify a wide gap in medical treatment served to the nation and raise substantial and efficient interest in the medical economy on the national basis. In practicing treatment according to these guidelines, it will be necessary to evaluate their therapeutic efficacy, and revise or add necessary supplements to them as required in the future.

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Acyclic retinoid inhibits angiogenesis by suppressing the MAPK pathway

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Acyclic retinoid (ACR) is currently under clinical trial as an agent to suppress the recurrence of hepatocellular carcinoma (HCC) through its ability to induce apoptosis in premature HCC cells. ACR has an anticancer effect *in vivo* as well, although it shows weak apoptosis-inducing activity against mature HCC cells, suggesting the existence of an additional action mechanism. In this study, we investigated the antiangiogenic activity of ACR. ACR inhibited angiogenesis within chicken chorioallantoic membrane (CAM) in as similar a manner as all-*trans* retinoic acid (atRA). Although suppression of angiogenesis by atRA was partially rescued by the simultaneous addition of angiopoietin-1, suppression of angiogenesis by ACR was not rescued under the same condition at all. Conversely, although suppression of angiogenesis by ACR was partially inverted by the simultaneous addition of vascular endothelial growth factor (VEGF), suppression of angiogenesis by atRA was not affected under the same condition. These results suggested that mechanisms underlying the suppression of angiogenesis by ACR and atRA were different. ACR selectively inhibited the phosphorylation of VEGF receptor 2 (VEGFR2) and of extracellular signal-regulated kinase (ERK) without changing their protein expression levels, and inhibited endothelial cell growth, migration, and tube formation. The inhibition of the phosphorylation of ERK, endothelial growth, migration, tube formation, and angiogenesis by ACR was rescued by the overexpression of constitutively active mitogen-activated protein kinase (MAPK). Finally, ACR, but not atRA, inhibited HCC-induced angiogenesis in a xenografted CAM model. These results delineate the novel activity of ACR as an antiangiogenic through a strong inhibition of the VEGFR2 MAPK pathway.

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KEYWORDS: ACR; HCC; MAPK pathway; phosphorylation; tumor angiogenesis; VEGF receptor

Angiogenesis has an important role in tumor growth by supplying nutrients and providing a route for metastasis.[1] Therefore, tumor angiogenesis is a good target for the treatment of solid cancers. Tumor cells induce angiogenesis by producing and releasing several angiogenic factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and angiopoietins (Angs).[1] The VEGF/VEGF receptor (VEGFR) signaling pathway is essential for drawing endothelial cells from preexisting blood vessels and in stimulating their growth,[2] whereas the Ang/Tie2 signaling pathway is important for sustaining the interaction between endothelial and mural cells and stabilizing the vasculature.

Retinoids (vitamin A and its derivatives) are natural fat-soluble hormones, the biological effects of which are believed to be mediated, all or in part, by the modulation of target gene expression through two families of nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs).[3] Retinoids exert antitumor activity by modifying the transactivation of $p21^{CIP1}$, interferon receptor, and signal transduction and activator of transcription.[4,5] We previously reported that all-*trans* retinoic acid (atRA) inhibits angiogenesis on chorioallantoic membrane (CAM) through disruption of vascular remodeling by inducing Ang2 expression and suppressing Ang/Tie2 signaling.[6] Acyclic retinoid (ACR)

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is a synthetic retinoid and activates the RAR and RXR.[7] Oral administration of ACR for 12 months significantly reduced the incidence of post-therapeutic recurrence of hepatocellular carcinoma (HCC) compared with the placebo group.[8] In this study, ACR did not cause the typical toxic effects observed with conventional retinoids.[8] Now, ACR is under clinical trials as a chemopreventive drug against the recurrence of HCC. Nuclear receptor RXR in HCC is highly phosphorylated through the Ras-extracellular signal-regulated kinase (ERK) pathway, inactivated, and accumulates in the line as a dominant-negative receptor.[9,10] ACR inhibits the phosphorylation of RXR by inactivating the Ras-ERK pathway, recovering transactivation by retinoic acid, and induces apoptosis in human HCC cell lines.[9,10] ACR also has an anticancer effect *in vivo*.[11] However, it exerts only weak apoptosis-inducing activity against mature HCC cells *in vivo*. This result suggests an existence of an additional molecular mechanism underlying the anticancer effect of ACR. Therefore, we predicted that ACR might have antiangiogenic activity.

Herein, we found that in contrast to the antiangiogenic mechanism of atRA, ACR inhibited angiogenesis through the inhibition of the VEGF receptor mitogen-activated protein kinase (MAPK) pathway. Moreover, ACR suppressed HCC-induced angiogenesis in a xenografted CAM model. These results suggest that ACR will also be clinically useful as an antiangiogenic agent, in addition to its current usage as a chemopreventive agent.

MATERIALS AND METHODS

Reagents

Acyclic retinoid (2E,4E,6E,10E)-3,7,11,15-tetramethylhexadeca-2,4,6,10,14-pentaenoic acid) was provided by Kowa (Tokyo, Japan). AtRA was purchased from Sigma-Aldrich (St Louis, MO, USA). ACR was dissolved in ethanol and dimethyl sulfoxide (DMSO) to yield stock solutions of 10 mM and 1 M, respectively, whereas atRA was dissolved in ethanol to yield a stock solution of 17 mM.

Chicken CAM Assay

In vivo antiangiogenic activity of ACR and atRA was assessed by CAM assay as described previously.[12] In brief, fertilized Dekalb chicken eggs (Omiya Kakin, Saitama, Japan) were placed in a humidified egg incubator. After a 4.5-day incubation at 38°C, a 1% solution of methylcellulose containing ACR or atRA at various concentrations was loaded inside a silicon ring that was placed onto the surface of CAM. After a further incubation for 2 days, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid. Antiangiogenic responses were evaluated under a stereomicroscope and photographed with a $\times 7.25$ objective. Quantitative analyses were carried out with angiogenesis-measuring software (ver.2.0; KURABO, Osaka, Japan).[12]

Matrigel Plug Assay

Matrigel (BD Biosciences, Bedford, MA, USA) was mixed with 200 units/ml heparin (Nacalai Tesque, Kyoto, Japan), with and without 50 ng/ml VEGF (Pepro Tech, Rocky Hill, NJ, USA) and 5 μM ACR in 0.1% DMSO. The matrigel mixture was injected subcutaneously into 5-week-old female C57BL/6 mice (Charles River, Yokohama, Japan). The mice were killed 7 days later. The matrigel plugs were removed and fixed in 4% paraformaldehyde for 4 h, dehydrated through a graded ethanol series, and embedded in paraffin (Nacalai Tesque). Vertical sections (5 μm) were mounted on slides and stained with hematoxylin and eosin, and observed under an inverted microscope (model DM IRB, Leica Microsystems, Wetzlar, Germany).

Cell Cultures

Human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells were cultured as described.[12] HepG2 cells, human HCC, were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum.

Transfection and Luciferase Assay

Transfection into HUVECs was carried out using a combination of LipofectAMINE 2000 Plus reagent (Invitrogen) and a constitutively active MAPK kinase vector (1.5 μg each per 35-mm dish).[13]

Western Blotting Analysis

After rinsing several times with TBS (20 mM Tris–HCl, 137 mM NaCl), cells were lysed in 1% Triton X-100 in 20 mM HEPES, pH 6.8, containing Complete protease inhibitor cocktail (1 tablet per 50 ml; Roche, Indianapolis, IN, USA), 1 mM EDTA, 1 mM PMSF, and 0.5 mM Na_3VO_5, and directly subjected to western analysis using phospho-VEGFR2-specific antibodies (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA), phospho-FGFR1-specific antibodies (1:1000 dilution; Cell Signaling Technology), or phospho-ERK-specific antibodies (1:2000 dilution, Cell Signaling Technology). Cell lysates were also subjected to western analysis using antibodies to VEGFR2, FGFR1, and ERK. Immunoreactive bands of proteins were detected with ECL-Plus chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

In Vitro Tube Formation Assay

Tube formation by HUVECs on matrigel was assessed as described previously.[14] Unpolymerized matrigel (Becton Dickinson, Bedford, MA, USA) was diluted to a final concentration of 5 mg/ml with MCDB-131 medium, aliquoted 150 μl each into 24-well plates, and allowed to polymerize for 30 min at 37°C. HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. Two days later, HUVECs were seeded onto the polymerized gel at 2×10^5 cells/well; thereafter, 100 ng/ml VEGF, 1 μM, 5 μM, and 10 μM ACR and/or atRA were added, and incubated for 6 h. *In vitro* tube formation was examined under a phase-contrast microscope and photographed with a × 10 objective.

HCC-Induced Angiogenesis in a Xenografted CAM Model

Hepatocellular carcinoma-induced angiogenesis in a xenografted CAM model was assessed as previously described.[15,16] HepG2 cell suspensions with or without 5 μM ACR or atRA were delivered at 4×10^5 cells per embryo onto the top of the CAM on day 8 using a gelatin sponge, called Gelform (Pfizer, New York, NY, USA) implant. After a further 4-day incubation, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid. Antiangiogenic responses were evaluated under a stereomicroscope and photographed with a × 25.

Statistical Analysis

Data are expressed as means ± s.d. Statistical significance was assessed by one-way analysis of variance, followed by Shaffer's *t*-test.

RESULTS

Comparison Between the Effects of ACR and atRA on Blood Vessel Formation in CAM

To determine whether ACR could inhibit *in vivo* angiogenesis, we carried out CAM assay (Figure 1). The formation of intricate vascular networks, developing within control CAM

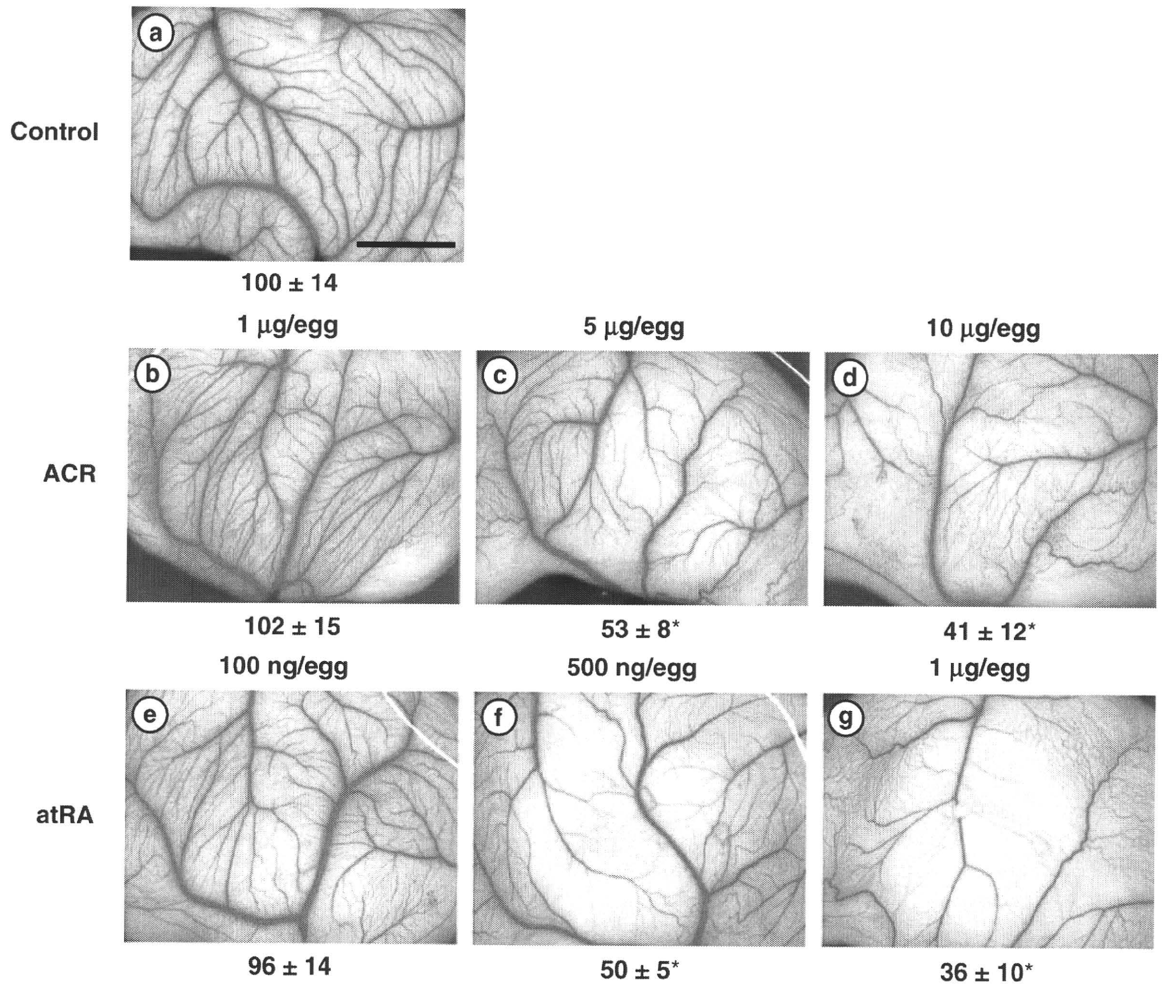

Figure 1 Suppression of *in vivo* angiogenesis in CAM by ACR and atRA. The 4.5-day-old CAMs were treated with ACR and atRA for 48 h, and then patterns of angiogenesis were photographed. Panel (**a**), vehicle (1% ethanol plus 1% DMSO); panel (**b**), 1 μg/egg ACR; panel (**c**), 5 μg/egg ACR; panel (**d**), 10 μg/egg ACR; panel (**e**), 100 ng/egg atRA; panel (**f**), 500 ng/egg atRA; panel (**g**), 1 μg/egg atRA. Scale bar, 5 mm. Total numbers of branches of blood vessels were analyzed with angiogenesis-measuring software and are shown under each panel. A total of 12 eggs (6 eggs per experiment × 2 experiments) were evaluated and representative results are shown. An asterisk indicates a significant difference ($P < 0.05$) from the control.

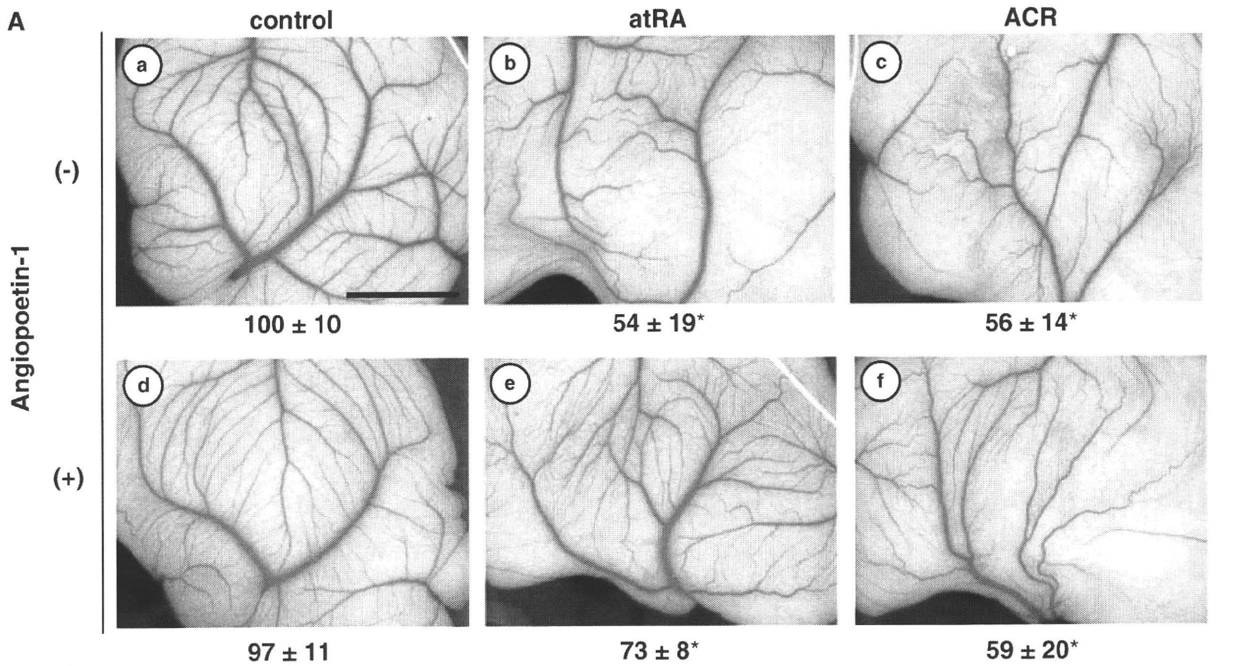

Figure 2 The antiangiogenic effect of atRA, but not ACR, was rescued by simultaneous treatment with Ang1 in CAM. (**A**) The 4.5-day-old CAMs were treated with ACR and atRA for 48 h and then patterns of angiogenesis were photographed. Panel (**a**), vehicle (1% ethanol plus 1% DMSO); panel (**b**), 500 ng/egg atRA; panel (**c**), 3 μg/egg ACR; panel (**d**), vehicle plus 300 ng/egg human recombinant Ang1; panel (**e**), 500 ng/egg atRA plus 300 ng/egg human recombinant Ang1; panel (**f**), 3 μg/egg ACR plus 300 ng/egg human recombinant Ang1. Scale bar, 5 mm. Total numbers of branches of blood vessels were analyzed with angiogenesis-measuring software and are shown under each panel. A total of 18 eggs (6 eggs per experiment × 3 experiments) were evaluated and representative results are shown. An asterisk indicates a significant difference ($P<0.05$) from the control. This result shows the representative result from three independent experiments, all of which gave similar results.

(Figure 1, panel a), was suppressed with ACR in a dose-dependent manner at concentrations of 1–10 μg/egg (0.3–3.3 mM inside the ring) (Figure 1, panels b–d) and with atRA in a dose-dependent manner at about 10 times lower concentrations of 100–1000 ng/egg (33–333 μM) (Figure 1, panels e–g). Although the inhibition of angiogenesis with atRA was partially rescued by simultaneous treatment with Angl at a concentration of 300 ng/egg as consistently as we reported previously[6] (Figure 2A, panel e), inhibition of angiogenesis with ACR was not rescued with Angl at all (Figure 2A, panel f). Furthermore, although atRA stimulated the transactivation activity of the *Ang2* promoter twofold (Supplementary Figure 1, column 2), ACR hardly showed such an activity (Supplementary Figure 1, columns 3 and 4). On the other hand, inhibition of angiogenesis with ACR, but not with atRA, was rescued by simultaneous treatment with VEGF (compare Figure 3A, panels e and f). To determine whether ACR might inhibit VEGF-induced blood vessel formation *in vivo*, we examined the effect of ACR in the matrigel plug assay (Figure 3B). Invasion of cells into gels was observed in the control matrigel that contained VEGF without ACR (panel a). When ACR was included in the matrigel at a concentration of 5 μM, the VEGF-induced invasion of cells was inhibited by about 54% (panel b).

Effect of ACR and atRA on Endothelial Cell Growth, Migration, and Tube Formation

We investigated the molecular mechanism by which ACR inhibited angiogenesis. First, we compared the effect of ACR and atRA on vascular endothelial cells. ACR (5 μM) suppressed the growth, migration, and tube formation (Figure 4A, lane 2, closed column; Figure 4B, lane 2, closed column; Figure 4C, panel b, respectively). These suppressive effects by ACR were, all or in part, rescued by overexpressing a constitutive active *MEK* gene (Figure 4A, lane 2, open column; Figure 4B, lane 2, open column; Figure 4C, panel e, respectively). Conversely, atRA did not suppress, rather it enhanced all of them (Figure 4A, lane 3, closed column; Figure 4B, lane 3, closed column; Figure 4C, panel c, respectively).

ACR Suppressed Phosphorylation of VEGFR2 and ERK

Next, we examined the effect of ACR and atRA on the phosphorylation of angiogenic growth factor receptors expressed by endothelial cells. As seen in the upper panel of Figure 5a, induction of phosphorylated 230 kD VEGFR2 after VEGF treatment was blocked to about 20% by pretreatment with 5 μM ACR for 24 h (compare lanes 4 with 5). In contrast, pretreatment with 5 μM atRA for 24 h did not block the

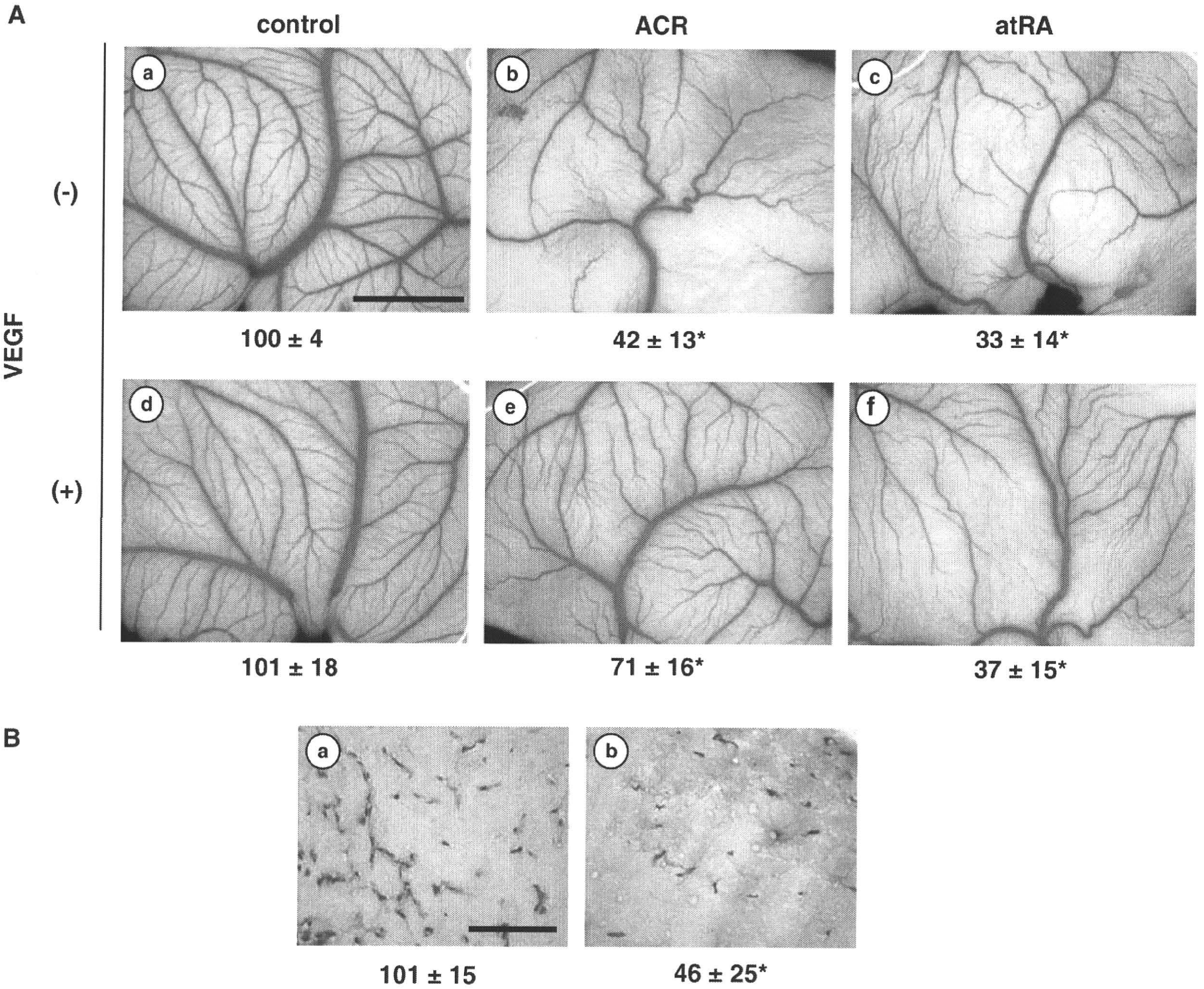

Figure 3 The antiangiogenic effect of ACR, but not of atRA, was rescued by simultaneous treatment with VEGF in CAM (**A**). The 4.5-day-old CAMs were treated with ACR and atRA for 48 h and then patterns of angiogenesis were photographed. Panel (**a**), vehicle (1% ethanol plus 1% DMSO); panel (**b**), 3 μg/egg ACR; panel (**c**), 500 ng/egg atRA; panel (**d**), vehicle plus 1 ng/egg mouse recombinant VEGF; panel (**e**), 3 μg/egg ACR plus 1 ng/egg mouse recombinant VEGF; panel (**f**) 500 ng/egg atRA plus 1 ng/egg mouse recombinant VEGF. Scale bar, 5 mm. Total numbers of branches of blood vessels were analyzed with angiogenesis-measuring software and are shown under each panel. A total of 18 eggs (6 eggs per experiment × 3 experiments) were evaluated and representative results are shown. (**B**) Matrigel plug assay: matrigel plugs containing 50 ng/ml VEGF ± 5 μM ACR were implanted into mice subcutaneously. One week later, matrigel plugs were collected and stained with hematoxylin and eosin (panels a and b). Panel **a**, VEGF alone (control); panel **b**, VEGF plus ACR. Representative data from a total of nine micrographs (3 fields × 3 mice) are presented. Scale bar, 500 μm. The number of invading cells in each micrograph was counted and the relative values are presented as percentages under each photograph. An asterisk indicates a significant difference ($P < 0.05$) from the control. Panels **A** and **B** show representative results from two independent experiments, both of which gave similar results.

phosphorylation of VEGFR2 but rather increased it (compare lanes 4 with 6). Both ACR and atRA decreased the expression of VEGFR2 to about 70 and 60%, respectively, without VEGF treatment (compare lanes 1 with 2 and 3). However, this effect was not obvious in cells treated with VEGF (compare lanes 4–6). ACR did not affect the binding of VEGF to VEGFR2, nor did it affect *VEGF* mRNA levels (data not shown). On the other hand, pretreatment with ACR or atRA did not block the phosphorylation of FGFR1 but rather enhanced it (Figure 5b). Whereas ACR inhibited the phosphorylation of Ras, it did not inhibit the phosphorylation of Akt (Supplementary Figure 2). In addition, pretreatment with 5 μM ACR, but not with atRA, significantly inhibited the phosphorylation of ERK, which is induced downstream of VEGF stimuli (Figure 6a, lanes 5 and 6 in upper panel, respectively). The inhibition by ACR was inverted by the overexpression of constitutively active MAPK kinase in HUVECs (Figure 6b, lane 4 in upper panel).

Effect of ACR and atRA on HCC-Induced Angiogenesis in a Xenografted CAM Model

To confirm whether ACR and atRA have anti-HCC-induced angiogenic activity *in vivo*, we investigated the effect of ACR and atRA on HCC-induced angiogenesis in a xenografted

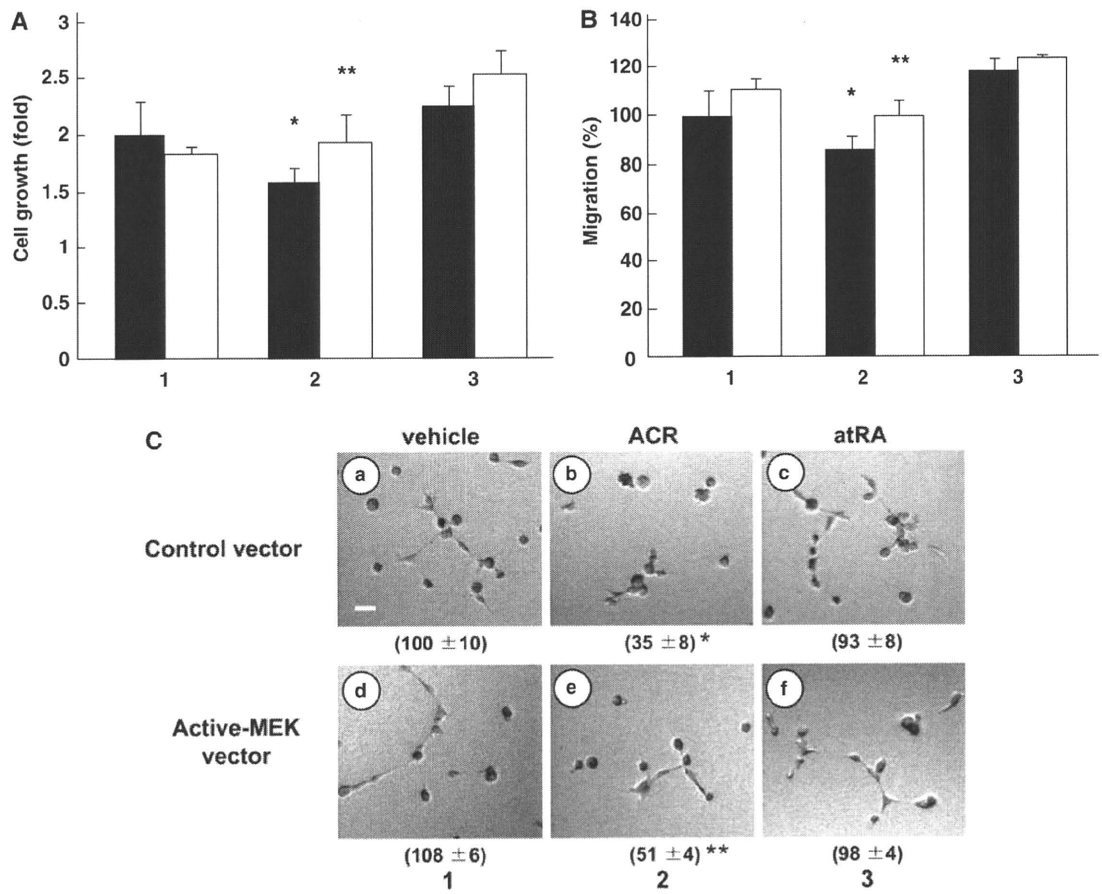

Figure 4 Effects of ACR and atRA on endothelial cell growth, migration, and tube formation. (**A**) HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. After 2 days, cells (1×10^5 cells) were seeded onto 3.5-cm dishes and incubated for another 2 days. They were then incubated for 48 h in α-MEM medium containing 10% fetal calf serum, 100 ng/ml VEGF, and 5 μM ACR or atRA. Cells were counted and cell numbers are plotted as ploidy relative to values for untreated control cells at the start of incubation with ACR or atRA. Values represent means ± s.e. ($n=2$). Lane 1, vehicle (0.1% ethanol); lane 2, ACR; lane 3, atRA. Closed columns, cells overexpressing control vector; open columns, cells overexpressing constitutively active MAPK kinase. A single asterisk indicates a significant difference ($P<0.05$) from the control (lane 1, closed column) and double asterisks indicate a significant difference ($P<0.05$) between samples with or without the overexpression of constitutively active MAPK kinase. (**B**) HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. After 2 days, cells were wounded with a tip of pipette and incubated for 12 h in α-MEM medium containing 2.5% fetal calf serum, 100 ng/ml VEGF, and 5 μM ACR or atRA. The numbers of cells that migrated into the denuded area were counted and are plotted as percentages relative to values for untreated control cells. Values represent means ± s.e. ($n=2$). Lane 1, vehicle (0.1% ethanol); lane 2, ACR; lane 3, atRA. Closed columns, cells overexpressing control vector; open columns, cells overexpressing constitutively active MAPK kinase. A single asterisk indicates a significant difference ($P<0.05$) from the control (lane 1, closed column) and double asterisks indicate a significant difference ($P<0.05$) between samples without or with the overexpression of constitutively active MAPK kinase. (**C**) HUVECs were transfected with a constitutively active MAPK kinase-expressing vector. After 2 days, cells were seeded onto polymerized matrigel at 2×10^5 cells/well. Thereafter, 100 ng/ml VEGF and 5 μM ACR or atRA were added, and incubated for 6 h. Patterns of tube formation were photographed. Scale bar, 100 μm. Panels (**a–c**), cells overexpressing control vector; panels (**d–f**), cells overexpressing constitutively active MAPK kinase. Panels (**a** and **d**), vehicle (0.1% ethanol) plus 100 ng/ml VEGF; panels (**b** and **e**), 5 μM ACR plus 100 ng/ml VEGF; panels (**c** and **f**), 5 μM atRA plus 100 ng/ml VEGF. The numbers of branches in each micrograph were counted and the relative values are presented as percentages under each photograph. A single asterisk indicates a significant difference ($P<0.05$) from the control (panel **a** *vs* panel **b**) and double asterisks indicate a significant difference ($P<0.05$) between samples with or without the overexpression of constitutively active MAPK kinase (panel **b** *vs* panel **e**). Panels **A–C** show representative results from two independent experiments, both of which gave similar results.

CAM model (Figure 7). Although 5 μM ACR inhibited HCC-induced angiogenesis by 37% (panel b), the same concentration of atRA did not show any inhibition at all (panel c). This result suggested that ACR, but not atRA, may prevent the recurrence of HCC in part through the inhibition of cancer angiogenesis.

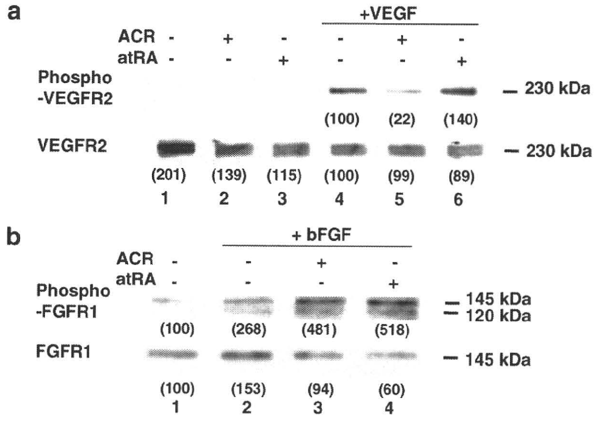

Figure 5 Effects of ACR and atRA on phosphorylation of growth factor receptors. After HUVECs had been incubated for 24 h with or without 5 μM ACR or atRA in medium containing 2.5% serum, cells were stimulated with either 100 ng/ml VEGF (panel **a**) or 50 ng/ml bFGF (panel **b**) for 5 min, and then lysed immediately. The amounts of each phosphorylated receptor (each upper bands), as well as whole amounts of each receptor (each lower bands), were assessed as described in the Materials and methods section. Panels **a** and **b** show representative results from two independent experiments, both of which gave similar results.

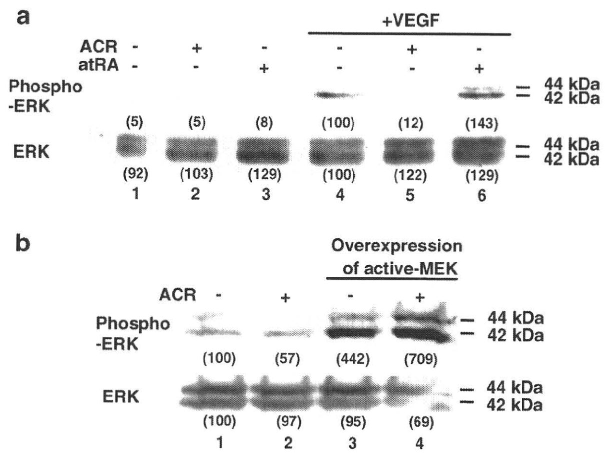

Figure 6 Effect of ACR on phosphorylation of ERK. (**a**) After HUVECs had been incubated for 24 h with or without 5 μM ACR or atRA in medium containing 2.5% serum, cells were stimulated with 100 ng/ml VEGF for 5 min and then lysed immediately. (**b**) HUVECs were transfected with a constitutively active *MEK* gene. The day after transfection, the medium was changed and cells were treated with 5 μM ACR and lysed immediately. The amounts of each phosphorylated ERK (upper bands), as well as whole amounts of ERK (lower bands), were assessed as described in the Materials and methods section.

DISCUSSION

In this paper, we have shown an antiangiogenic activity of ACR and its underlying molecular mechanism, differing from that of atRA. Although the relative antiangiogenic activity of ACR was about 10 times weaker than that of atRA at the same concentrations (Figure 1), ACR showed much stronger inhibition in endothelial cell growth, migration, and tube formation than atRA (Figure 4) because of suppression in the VEGF-MAPK pathway (Figures 5 and 6). ACR suppressed the phosphorylation of VEGFR2 (Figure 5a, lane 5). On the other hand, atRA induced the phosphorylation of VEGFR2 (Figure 5a, lane 6). This might be because of the induction of VEGF by atRA as reported previously.[17] ACR did not affect the levels of *VEGF* mRNA and the transactivation of the *VEGF* promoter (data not shown). ACR slightly inhibited the phosphorylation of VEGFR1 at 300 μM but not at all at 30 μM (Ishibashi *et al*, unpublished data). ACR (1 or 10 μM) did not inhibit other tyrosine kinases (for example, EGFR, FGFR3, FLT3, IGF1R, MET, PDGFR-α, PDGFR-β, and TRKB) (Ishibashi *et al*, unpublished data). Moreover, ACR inhibited the phosphorylation of Ras but not the phosphorylation of Akt (Supplementary Figure 2). Therefore, we speculate that ACR may selectively interfere with the phosphorylation of VEGFR2 after Ras activation, although it is not clear how ACR does this; the underlying detailed molecular mechanism(s) remain to be elucidated. ACR and atRA enhanced the phosphorylation of FGFR1 (Figure 5b, lanes 3 and 4). This result might be because of the induction of bFGF by atRA as previously reported.[18] ACR might also induce bFGF, probably through its retinoid activity.

These results suggest that suppression of the recurrence of HCC by ACR was induced in part through its antiangiogenic property by directly suppressing endothelial growth, migration, and tube formation through inhibition of the VEGFR2-MAPK axis. On the other hand, atRA inhibits angiogenesis by a disruption of vascular networks through an increased expression of Ang2 and inhibition in the Ang/Tie2 pathway.[6] Compared with atRA, ACR has 1/10–1/100 weaker 'retinoid' activities.[19] It has a 1/10 weaker action on leukemia differentiation,[20] and a 1/100 weaker carcinogenic action in transgenic mice that express the dominant-negative form of retinoic acid receptor.[21,22] On the other hand, accumulating evidence shows that ACR, but not atRA, has apoptosis-inducing activity in HCC cells, as well as in smooth muscle cells and vascular neointima.[23,24] We found that ACR acts as either a kinase inhibitor or a phosphatase stimulator and prevents hyperphosphorylation of RXR,[9] and now VEGFR2. In this context, we speculate that ACR resembles geranylgeraniol in terms of its isoprenoid-like structure, which has been implicated in the modulation of many phosphorylation/dephosphorylation signaling.[25,26]

Hepatocellular carcinoma is a major cause of cancer mortality worldwide, especially in Southeast Asia and in sub-Saharan Africa.[27] The development of HCC is frequently associated with a chronic inflammation of the liver induced by persistent infection with hepatitis B virus or hepatitis C virus. The annual incidence rises to ~20–25% in cirrhotic patients who have undergone a potentially curative removal of primary HCC in Japan; the recurrence rate at 5 years after the curative treatment may exceed 70%. This high recurrence rate is not because of local recurrence or metastasis from the original lesion, but rather from a second primary lesion.[19]

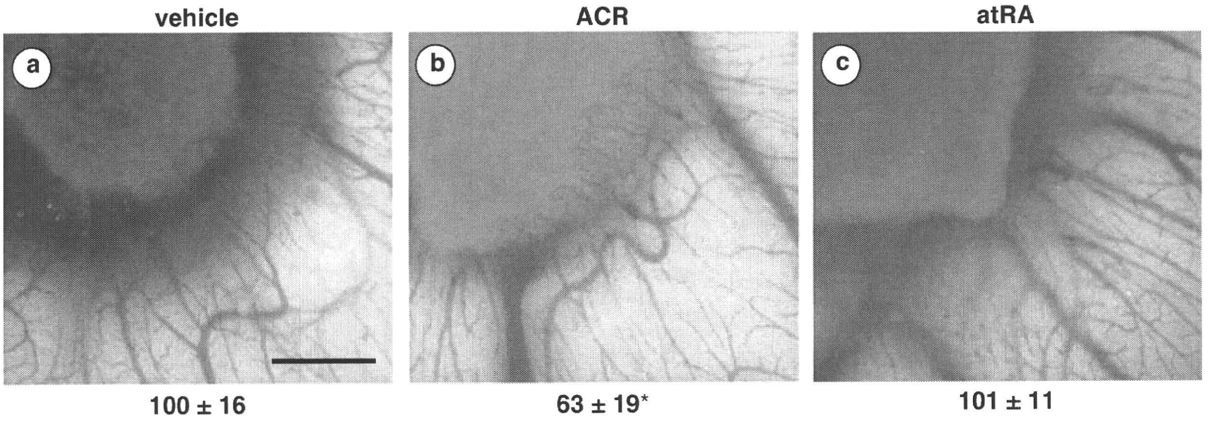

Figure 7 Effect of ACR on HCC-induced angiogenesis on CAM. HepG2 cell suspensions with or without 5 μM ACR or atRA were delivered at 4×10^5 cells per embryo onto the top of CAM on day 8 using a gelatin sponge implant. After a further 4-day incubation, a fat emulsion was injected into the chorioallantois, so that the vascular networks stood out against the white background of the lipid, and patterns of angiogenesis toward the implant were photographed. Panel (**a**), vehicle (1% EtOH); panel (**b**), 5 μM ACR; panel (**c**), 5 μM atRA. Scale bar, 1 mm. Total numbers of branches of blood vessels were counted and the relative values are presented as percentages under each photograph. An asterisk indicates a significant difference ($P < 0.05$) from the control. This result shows the representative result from two independent experiments, both of which gave similar results.

In HCC tissues, RXR-α is constitutively phosphorylated by the activation of MAPK, resulting in a loss of its function and accumulation of inactive RXR-αs inside cells as dominant-negative inhibitors.[9,10] Therefore, phosphorylation of RXR-α causes a reduction in transactivation through a RAR/RXR complex.[9,28] ACR inhibits the phosphorylation of RXR-α and restores the function of RXR-α, and thereby transactivation through the RAR/RXR complex with endogenous retinoic acid. Retinoids are thought to activate the transcription of cell cycle inhibitor p21^{CIP1} by RAR[29] and by apoptosis-inducer tissue transglutaminase in HCC.[19] However, 5 μM ACR hardly induces endothelial cell death (Figure 4A) and expression of *p21*^{CIP1} mRNA levels in endothelial cells (data not shown). We found that phosphorylation of RAR/RXR was associated with the growth of endothelial cells and that ACR inhibited this phosphorylation (Supplementary Figure 3). As this phosphorylation of RAR/RXR coincided with the activation of VEGFR2 and Ras and as we had already found that RAR/RXR phosphorylation was induced by the overexpression of active MEK in cancer cells,[10] we speculate that phosphorylation of RAR/RXR would occur downstream of the VEGF/VEGFR axis in growing endothelial cells.

Acyclic retinoid suppressed both angiogenesis and HCC-induced angiogenesis in a xenografted CAM model (Figure 1, panels b–d and Figure 7, panel b, respectively). In this model, ACR did not affect the preexisting blood vessels on CAM. These results suggest that ACR did not affect the mature blood vessel and only affected neovasculature formation, including both normal angiogenesis and tumor angiogenesis. On the other hand, atRA suppressed angiogenesis but failed to suppress the tumor angiogenesis on CAM (Figures 1 and 7, panels e–g and panel c, respectively), suggesting that atRA inhibits angiogenesis in the embryonic stage, but not tumor-induced angiogenesis, and addressing the superiority of ACR as a promising antitumor angiogenesis agent. The dose of ACR used in this experiment (5 μM) is higher than the maximal blood concentration of ACR in clinical use (1 μM) (Ishibashi *et al*, unpublished data). However, we believe that liver tissue has higher doses of ACR than does the blood level because of the accumulation of ACR in the liver. These results suggest that one of the mechanisms of ACR action to prevent recurrence of HCC is its antiangiogenic activity.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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