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An antioxidant effect by acyclic retinoid suppresses liver tumor in mice

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ABSTRACT

The mechanisms of prevention of the development of liver cancer by NIK-333, an acyclic retinoid (ACR), were investigated. The transgenic mice expressing the dominant negative form of retinoic acid receptor α (RARE mice), that produce reactive oxygen species and lead to development of liver tumor were used. The effect of NIK-333 on hepatocarcinogenesis in RARE mice was studied. The RARE mice were examined after feeding 0.03% and 0.06% NIK-333 diets at 12 months of age. In the mice fed 0.06% NIK-333 diet, tumor incidence was greatly suppressed, compared to that of wild type mice (0/9 versus 5/9, $P < 0.05$), but not in the mice fed 0.03% NIK-333 diet. In addition, expression of cytochrome p450 4a14 and acyl-CoA oxidase was normalized, and the percentages of positive cells for 8-hydroxy-2′-deoxyguanosine, 4-hydroxy-2-nonenal and proliferating cell nuclear antigen were decreased. Furthermore, expression of β-catenin and cyclin D1 was also depressed. These data suggest that NIK-333 suppressed liver tumor in association with repression of oxidative stress.

1. Introduction

Retinoids, vitamin A (retinol) metabolites, are physiological regulators of a large number of essential biological processes including embryonic development, vision, reproduction, bone formation, metabolism, hematopoiesis, differentiation, proliferation and apoptosis [1]. A strong relationship between vitamin A deficiency and cancer development has been known. Vitamin A deficiency in experimental animals has been associated with a higher incidence of cancer [2]. Epidemiological studies indicated that individuals with a lower dietary vitamin A intake are at a higher risk to develop cancer [3]. In addition, retinoids were shown to suppress carcinogenesis in experimental animals [4], and in head and neck area cancer patients and in xeroderma pigmentosum patients [5]. Indeed, all-*trans*-retinoic acid (atRA), a natural vitamin A metabolite, was approved by the FDA for the treatment of patients with acute promyelocytic leukemia

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Abbreviations: ACR, acyclic retinoid; LCAD, long-acyl-CoA dehydrogenase; VLCAD, very long-acyl-CoA dehydrogenase; AOX, acly-CoA oxidase; CYP4a14, cytochrome p4504a14; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; 4-HNE, 4-hydroxy-2-nonenal; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehydes-3-phosphate dehydrogenase

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(APL). A recent report has indicated that high serum levels of retinol are associated with a decreased risk of hepatocellular carcinoma (HCC) in China [6]. These findings have led to the hypothesis that physiological levels of retinoids guard the organism against the development of premalignant and malignant lesions.

A extensive research effort has been dedicated to elucidate the molecular and cellular mechanism of retinoid action. The pleiotropic effects of retinoids are mediated by retinoic acid receptor (RAR) and retinoid X receptor (RXR). Each type of nuclear retinoid receptor includes three subtypes: α, β and γ. In our recent studies, RAR α dominant negative form (RARE) caused steatohepatitis and liver tumors [7,8]. In the livers of these mice, mitochondrial β-oxidation was depressed, peroxisomal β-oxidation was increased, and microsomal ω-oxidation was also increased. Formation of H_2O_2 and 8-hydroxy-2′-deoxyguanosine (8-OHdG) was increased, being suggesting that oxidative stress plays an important role in hepatocarcinogenesis. Importantly, RA is supposed to suppress liver cancer by repressing oxidative stress.

Acyclic retinoid (ACR), NIK-333, a novel synthetic retinoid, inhibits the growth of HCC cells *in vitro* and *in vivo* [9,10]. In a clinical study, the administration of ACR to the patients that had been treated for HCC prevented the occurrence of secondary HCC [11]. ACR induces apoptosis in human HCC cell lines [12], and ACR also causes an arrest of the cell cycle in G0–G1, in association with increased p21 protein and decreased cyclin D1 in human HCC cells [10].

Approximately 170 million people in the world are chronically infected with hepatitis C virus (HCV), and chronic HCV infection is the main cause of liver cirrhosis and HCC [13]. The molecular mechanism of HCV pathogenesis remains unclear, however, oxidative stress has emerged as a key player in the development and the progression of many pathological conditions, including HCV-induced liver diseases and non-alcoholic steatohepatitis (NASH) [13,14]. Therefore, suppression of oxidative stress may be very important as chemoprevention for liver diseases such as HCV-related liver diseases and NASH. In the present study, we examined the chemopreventive action of ACR on HCC.

2. Materials and methods

2.1. Animals

The RARE transgenic mice were developed as previously reported [7,8]. For the present study, we concentrated on mice of the TG.943 line, which demonstrated the greatest abnormalities on initial screening for histological changes although the mice of other lines developed similar histological abnormalities. At 1 month of age, the male mice were divided into four groups: the mice of RARE control, RARE 0.03% NIK-333, RARE 0.06% NIK-333, and wt control. The RARE control, RARE 0.03% NIK-333, and RARE 0.06% NIK-333 were RARE transgenic mice, and wt control were wild type mice. The mice of RARE control and wt control were fed a CE-2 diet (CLEA Japan, Tokyo, Japan), RARE 0.03% NIK-333 were fed 0.03% NIK-333-containing CE-2 diet, and RARE 0.06% NIK-333 were fed a 0.06% NIK-333-containing CE-2 diet. The rationale for the

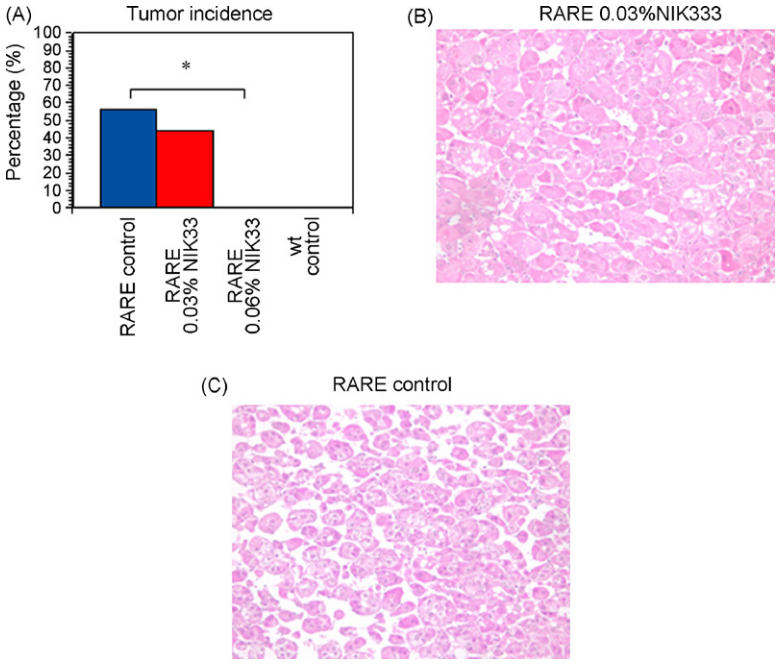

Fig. 1 – Incidence of tumor and histopathology. Male RARE mice were fed a control diet ($n = 9$), or 0.03% ($n = 9$) or 0.06% NIK-333 ($n = 9$). A wt control group ($n = 9$) was also included. Animals were examined for liver lesions at 12 months of age. (A) The percentages of tumor incidence in four groups. The incidence of tumor development was compared using the Fisher's exact test. $^{*}P < 0.05$ between two groups. (B) Hepatocellular adenoma in the liver of the mouse (268) of RARE 0.03% NIK-333 (H&E staining, magnification: 200×). (C) Well differentiated HCC in the liver (746) of RARE control (H&E staining, magnification: 200×).

doses selection of NIK-333 was that similar doses of NIK-333 inhibited hepatocarcinogenesis induced by chemical agents [15,16]. Each group includes nine mice, respectively. All the mice were sacrificed at 12 months of age and examined for tumor development, and molecular and immunohistochemical analyses. The mice were kept under pathogen-free conditions, and were maintained in a temperature-controlled room with a 12 h light/dark illumination cycle. Animals received humane care in accordance with study guidelines established by the Tottori University Subcommittee on Laboratory Animal Care.

2.2. *Histological analysis of tissues, tumors, and hepatocyte proliferation*

Histological examination was done by the tissues fixed in 4% paraformaldehyde. Hepatic tumor was categorized to hepatocellular adenoma and hepatocellular carcinoma (HCC) [17,18]. Briefly, hepatocellular adenoma is a discrete lesion that compresses adjacent parenchyma. It is composed of well differentiated cells that may be eosinophilic, basophilic, or vacuolated. HCC is diagnosed by a distinct trabecular or adenoid pattern. The distinction between hepatocellular adenoma and HCC is relative and depends on the perceived degree of cytologic differentiation. The immunohistochemical analysis was done as follows: the sections were incubated with 0.3% H_2O_2 in methanol for 30 min. They were heated in 10 mmol/l sodium citrate buffer (pH 6.0) at 600 W for 15 min in a microwave oven. After washing, they were incubated with the following primary antibodies: a monoclonal anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) (NOF Corp., Tokyo, Japan), a monoclonal anti-4-hydroxy-2-nonenal (4-HNE) (NOF Corp., Tokyo, Japan) and a monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody (NCL-PCNA, Novacastra Laboratories, Newcastle, UK) at 4 °C overnight. Then, they were incubated with biotin-conjugated anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) for 60 min. Immunoreactive cells were visualized using a Vectastain ABC-PO Kit (Vector Laboratories).

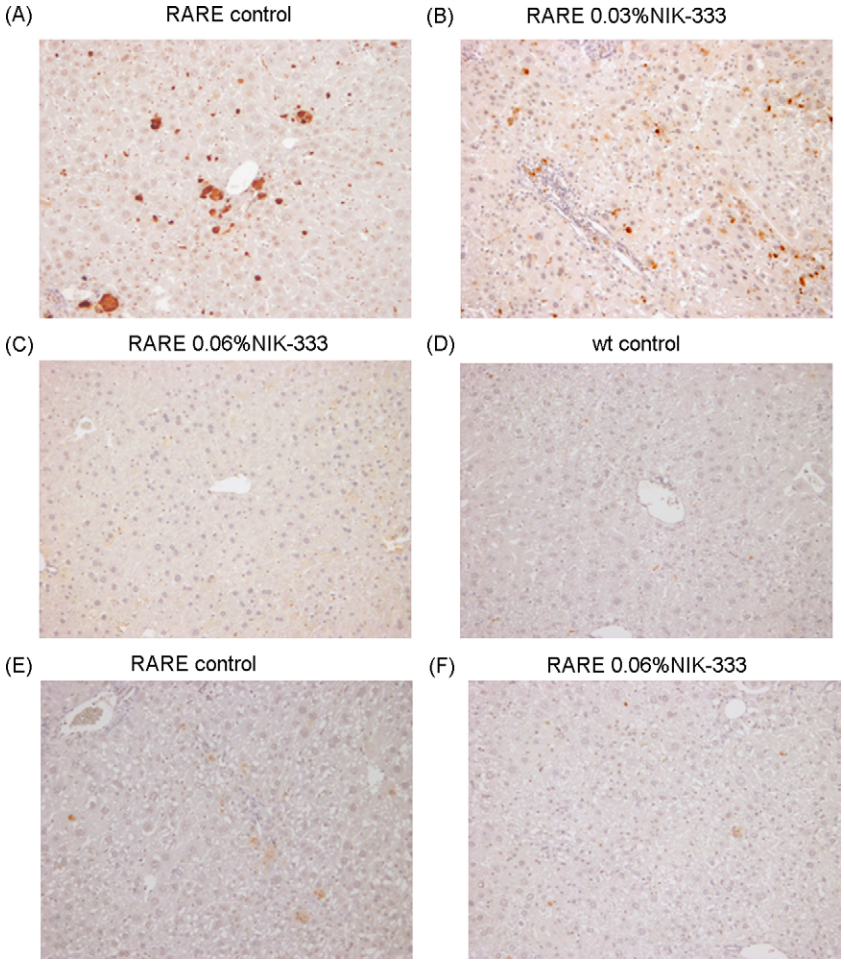

Fig. 2 – Histochemical study of the livers. Many 8-OHdG-positive hepatocytes were present in the liver of the mouse (745) of RARE control (magnification: 200×). (B) The smaller number of hepatocytes were stained with 8-OHdG antibody in the liver of the mouse (274) of RARE 0.03% NIK-333, compared with those of RARE control (magnification: 200×). (C) The positive cells for 8-OHdG were not observed in the liver of mouse (259) of RARE 0.06% NIK-333 (magnification: 200×). (D) The positive cells for 8-OHdG were not observed in the liver of mouse (242) of wt control (magnification: 200×). (E) The 4-HNE-positive cell were scattered in the liver lobule of the mouse (310) of RARE control (magnification: 200×). (F) The 4-HNE-positive cell were not present in the liver of the mouse (761) of RARE 0.06% NIK-333 (magnification: 200×).

Table 1 – Primers of real-time RT-PCR

Genes	Forward	Reverse
CYP4a14	5′-CCACAGGACATGCAGATTAG-3′	5′-CACACAGAGCTCGGAAGACC-3′
AOX	5′-CCACAGGGACATGCAGATTAG-3′	5′-CACACAGAGCTCGGAAGACC-3′
LCAD	5′-GTGGACAGCTGTCTGCAG-3′	5′-CCCCCCCTTTTCCAGGTT-3′
VLCAD	5′-TGCAAGGGCTGTATGGCC-3′	5′-GGCACATGACCTTGCCAG-3′
β-Catenin	5′-GCAGATCTTGGACTGGACATTG-3′	5′-GCCGTATCCACCAGAGTGAA-3′
Cyclin D1	5′-CCCAACAACTTCCTCTCCTGCTA-3′	5′-GGCTTCAATCTGTTCCTGGC-3′
β-Actin	5′-CCACAGGATTCCATACCCAAGA-3′	5′-GACGGCCAGGTCATCACTATTG-3′

CYP4a14, cytochrome p450 4a14; AOX, acyl-coA oxidase; LCAD, long-acyl-CoA dehydrogenase; VLCAD, very long-acyl-CoA dehydrogenase.

The positive cells were expressed as the percentages of 1000 cells counted in 5 randomly selected fields per animal.

2.3. *Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR)*

Total RNA was extracted from liver tissues using ISOGEN (Nippongene Co., Toyama, Japan) and was digested with DNase (Nippongene). Complementary DNA (cDNA) was synthesized using the RNA LA PCR kit (TaKaRa Bio Inc., Kyoto, Japan). Real-time RT-PCR was done with LightCycler® using SYBR Green I Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The genes amplified were cytochrome p450 4a14 (CYP4a14), acyl-coA oxidase (AOX), long-acyl-CoA dehydrogenase (LCAD), very long-acyl-CoA dehydrogenase (VLCAD), β-catenin, cyclin D1 and β-actin. The primers were listed in Table 1. Reported copy numbers were the geometric average of five determinations derived from independent PCRs performed from the same cDNA. The values were reported as the normalized quotient (NQ), derived by dividing the copy number of each gene by the GAPDH copy number.

2.4. *Statistics*

Values were expressed as means ± S.D. Means were compared using the Mann–Whitney *U*-test. The incidence of tumor development was compared using the Fisher's exact test. A *P* value less than 0.05 was considered to be significant.

3. Results

3.1. *Tumor development*

The consumed amounts of the diet per mouse were similar among four groups. The body weights and the liver/body weights in mice fed a NIK-333 diet were almost the same as those in fed a CE-2 diet, respectively. At 12 months of age, 5 of 9 mice developed liver tumor in RARE control, and 4 of 9 mice

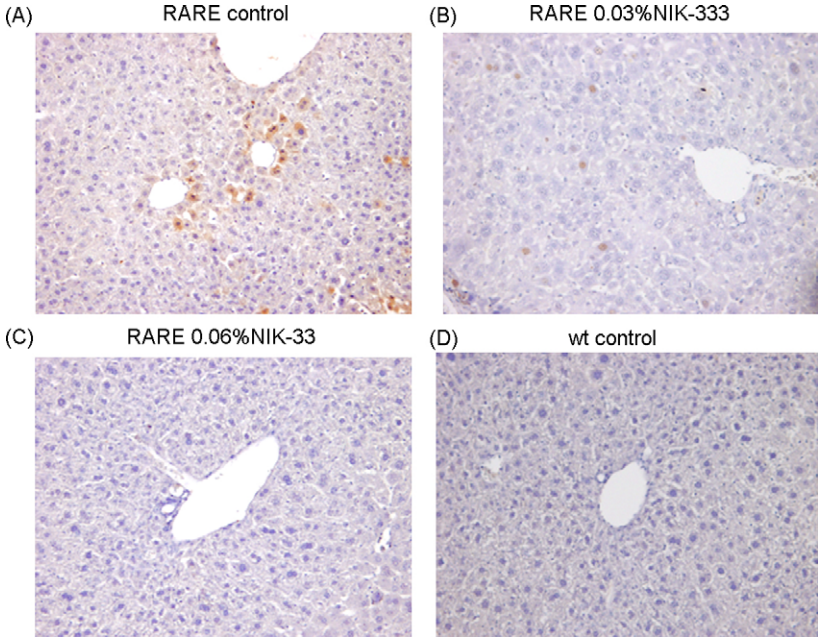

Fig. 3 – Expression of PCNA. (A) Many PCNA-positive cells were clustered in the liver of the mouse (762) of RARE control (magnification: 200×). (B) Fewer cells were stained faintly in the liver of the mouse (268) of RARE 0.3% NIK-333 (magnification: 200×). (C) Positive cells were not observed in the mouse (730) of RARE 0.06% NIK-333 (magnification: 200×). (D) Positive cells were not observed in the mouse (328) of wt control (magnification: 200×).

developed in RARE 0.03% NIK-333. On the other hand, no liver tumors developed in RARE 0.06% NIK-333 or wt control. The rate of liver tumor incidence in RARE control was higher than that in RARE 0.06% NIK-333 ($P < 0.05$, Fig. 1A). All the tumors were single, and the color of the tumor was whitish (data not shown). The sizes of tumor were around 5 mm, and no differences were observed between the groups (data not shown). The pathological analysis of a tumor in RARE 0.03% NIK-333 showed that eosinophilic cytoplasm was abundant, and was diagnosed as hepatocellular adenoma (Fig. 1B). In a tumor in RARE control, the tumor consisted of eosinophilic and acinar formed well differentiated HCC cells (Fig. 1C). Five tumors included one HCC and four hepatocellular adenoma in RARE control, and all the four tumors in RARE 0.03% NIK-333 were hepatocellular adenoma. Steatosis was not prominent in the mice in all the groups. In non-tumorous areas, some dysplasia was observed in the mice of RARE control and RARE 0.03% NIK-333, however, it was not observed in RARE 0.06% NIK-333 and wt control (data not shown).

3.2. *Oxidative stress*

Since in our previous report, we found that retinoic acid (RA) prevents onset of liver tumor through inhibition of oxidative stress [8], we examined the status of reactive oxygen species (ROS). First, levels of 8-hydroxyl-2′-deoxyguanosine (8-OHdG) were examined. In the liver of RARE control, 8-OHdG-positive cells were distributed in the liver lobule (Fig. 2A). In the liver of RARE 0.03% NIK-333, less 8-OHdG-positive cells were present (Fig. 2B). No 8-OHdG-positive cells were observed in the livers

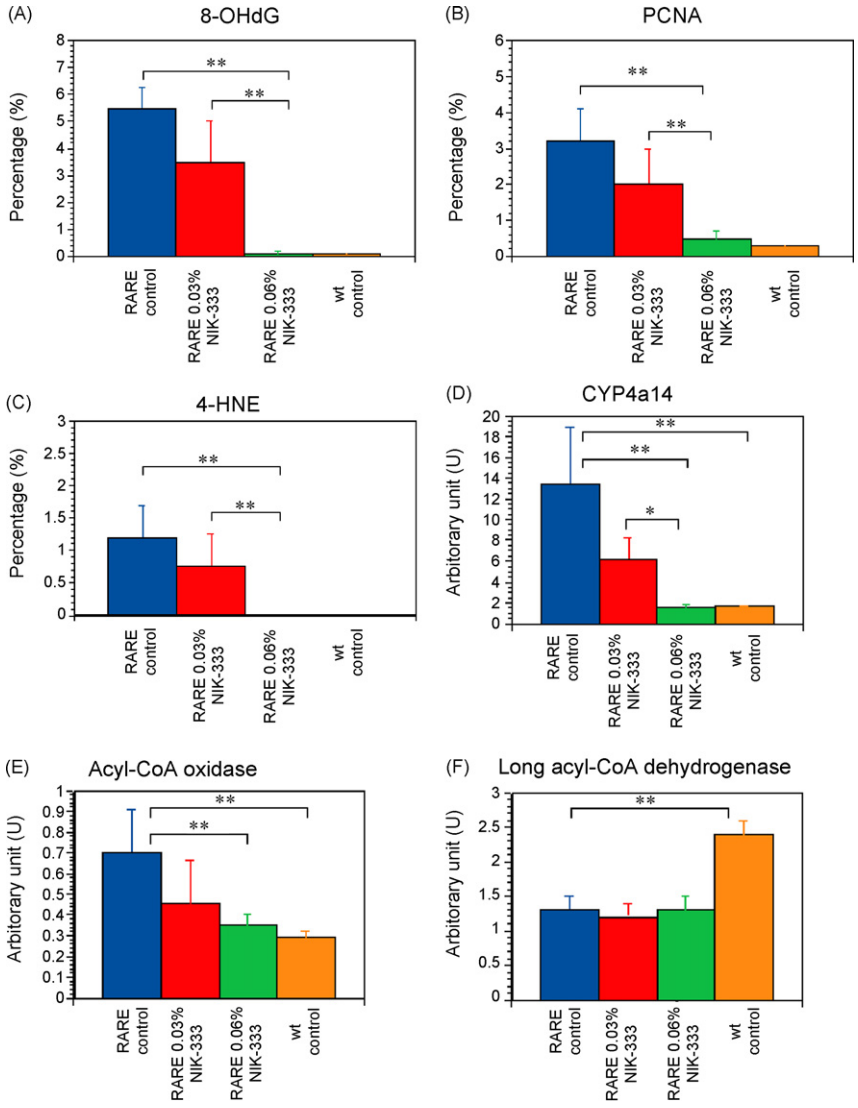

Fig. 4 – Oxidative stress, cell proliferation and expression of enzymes. The percentages of 8-OHdG-positive cells in four groups. Significance was calculated by the Mann–Whitney *U*-test. $^{**}P < 0.01$ between two groups. (B) The percentages of PCNA-positive cells in four groups. $^{**}P < 0.01$ between two groups. (C) The percentages of 4-HNE cells in four groups. $^{**}P < 0.01$ between two groups. (D) Expression level of CYP4a14. $^{**}P < 0.01$; $^{*}P < 0.05$ between two groups. (E) Expression level of AOX. $^{**}P < 0.01$ between two groups. (F) Expression level of LCAD. $^{**}P < 0.01$ between two groups. (G) Expression level of VLCAD. $^{*}P < 0.05$ between two groups. (H) Expression level of β-catenin. $^{*}P < 0.05$ between two groups. (I) Expression level of cyclin D1. $^{*}P < 0.05$ between two groups.

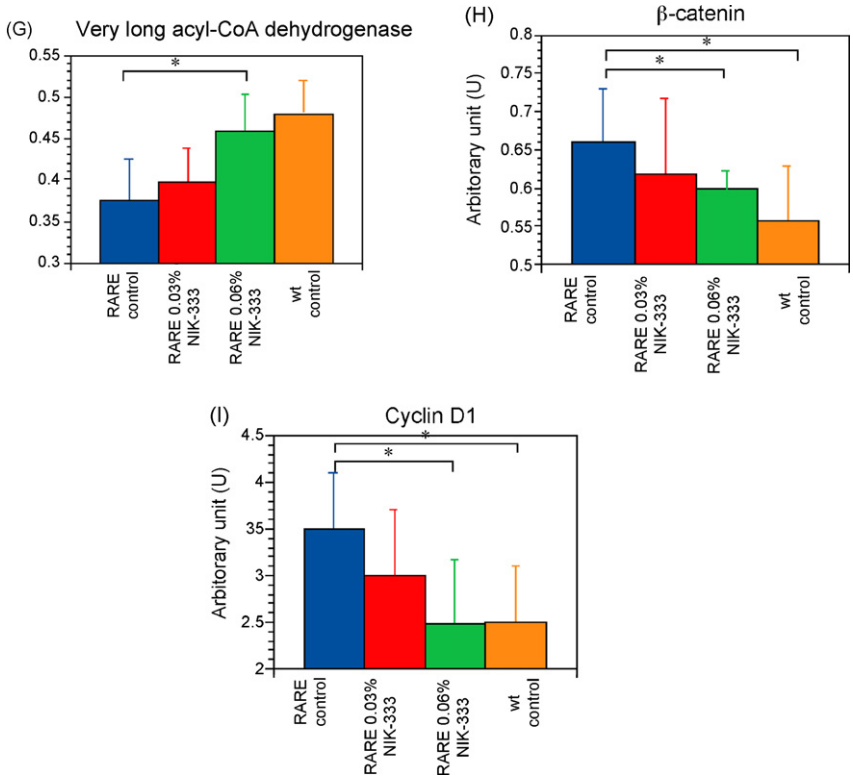

Fig. 4. (Continued).

of RARE 0.06% NIK-333 or wt control (Fig. 2C and D). The percentages of 8-OHdG-positive cells in RARE control and RARE 0.03% NIK-333 were greater than those in RARE 0.06% NIK-333 (Fig. 4A). Second, the 4-HNE-positive cells were scattered in the liver lobule in RARE control, however, the number of positive cells were not so many (Fig. 2E). On the other hand, the 4-HNE-positive cells were absent in the liver of RARE 0.06% NIK-333 (Fig. 2F). The rates of 4-HNE-positive hepatocytes in RARE control and RARE 0.03% NIK-333 were greater than that in RARE 0.06% NIK-333 (Fig. 4C). The PCNA-positive hepatocytes were increased in the mice in RARE control, compared with those of wt control (Fig. 3A and D). Feeding with 0.03% NIK-333 diet decreased the number of PCNA-positive cells (Fig. 3B), and that with 0.06% NIK-333 greatly suppressed the number of PCNA-positive cells (Figs. 3C and 4B). These data suggest that feeding 0.06% NIK-333 reduced the amount of ROS.

The molecular events were examined by real-time RT-PCR. Expression of CYP4a14 in RARE control was increased, compared with that in wt control ($P < 0.01$, Fig. 4D). Although feeding 0.03% NIK-333 did not reduced the expression of CYP4a14, feeding 0.06% NIK-333 significantly reduced that of CYP4a14 ($P < 0.05$). The expression levels of AOX was not altered by feeding 0.03% NIK-333, however, it was reduced by 0.06% NIK-333 ($P < 0.01$, Fig. 4E). These data suggest that microsomal ω-oxidation and peroxisomal β-oxidation were normalized by 0.06% NIK-333. These seemed to be the major causes of antioxidative effect of NIK-333. Expression of LCAD was decreased in the mice in RARE control ($P < 0.01$), however, the addition of NIK-333 did not increase its expression level (Fig. 4F). Expression of VLCAD in RARE 0.06% NIK-333 was significantly higher than that in RARE control ($P < 0.05$, Fig. 4G). Although steatosis is not prominent in four groups of mice at 12 months of age, these data suggest that feeding with NIK-333 reverses the abnormality of β-oxidation of fatty acids. Since in our previous study expression of β-catenin and cyclin D1 was increased [8], expression of these molecules was examined. Feeding 0.06% NIK-333 diet significantly depressed expression of β-catenin and cyclin D1 ($P < 0.05$, each, Fig. 4H and I). Thus, inactivation of Wnt/β-catenin signals may be one of responsive mechanisms of NIK-333 effect.

4. Discussion

In the present study, 0.06% NIK-333 suppressed the occurrence of liver tumor. Administration of NIK-333 decreased the numbers of positive cells for 8-OHdG, 4-HNE and PCNA, and normalized expression of CYP4a14 and AOX. These data suggest that downregulation of microsomal ω-oxidation and peroxisomal β-oxidation by NIK-333 decreased production of reactive oxygen species (ROS), and that reduction of ROS was associated with decreased cell proliferation. However, 0.03% NIK-333 did not decrease expression of CYP4a14 and AOX or ROS, and finally did not suppress the onset of liver tumor. The amount of RA that is two orders of magnitude greater than effective doses is necessary to overcome the dominant effect of RARE [19]. In our previous report, feeding the RARE mice on the high-RA diet containing 50.5 mg/kg all-*trans*-retinoic acid (atRA) for 12 months suppressed liver tumors [8]. The high-RA diet contains about 101-fold RA of standard diet. It was reported that NIK-333 induced the differentiation of HL-60

cells and acute promyelocytic leukemia cells at 10^{-6} mol/l while atRA induced differentiation at 10^{-7} mol/l [20]. Judging from this finding, the action of NIK-333 may be equivalent to 1/10 of atRA. Therefore, 0.06% NIK-333 (600 mg/kg NIK-333) may be equivalent to 60 mg/kg atRA. In the present study, 0.06% NIK-333 suppressed the onset of HCC, however, 0.03% NIK-333 did not. These findings are coincident with our previous report; 50 mg/kg atRA reversed the action of the dominant negative effect [8]. Therefore, NIK-333 has the similar effect as atRA in the RARE mice. Taken together with the data [15,16], NIK-333 exerts via retinoid receptors, suppressing hepatocarcinogenesis in RARE mice as well as chemically induced liver cancers.

In the present study, expression of mitochondrial enzymes involved in fatty acid β-oxidation such as LCAD was not changed by administration of NIK-333, however, feeding with 0.06% NIK-333 reversed expression of VLCAD. Steatosis was not observed at 12 months of age in the RARE mice [8], and the meaning of no alteration of these enzymes remains unclear. However, since the blockade of retinoid receptors caused many abnormalities in several organelles [8], these findings suggest that downstream signals of NIK-333 are mediated via other mechanisms than atRA.

In the present study, NIK-333 suppressed expression of CYP4a14, one of major fatty acid ω-hydroxylase in mouse. Recently, it has been reported that CYP4a11, the major fatty acid ω-hydroxylase in human is repressed by atRA [21]. CYP4a sub-family is induced by different compounds, e.g., fatty acids and peroxisome proliferators [22]. These effects are considered to be mediated via the peroxisome proliferators-activated receptor α (PPARα) [23]. In the RARE mice, expression of PPARα was increased [8]. How NIK-333 regulates PPARα and the mechanism of regulation of PPARα is the same as atRA remain to be clarified in the future.

In conclusion, NIK-333 suppressed liver tumor in association with reduction of oxidative stress.

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