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An acyclic retinoid, NIK-333, inhibits *N*-diethylnitrosamine-induced rat hepatocarcinogenesis through suppression of TGF-α expression and cell proliferation

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The present study was designed to determine the effects of NIK-333, a synthetic acyclic retinoid, on *N*-diethylnitrosamine (DEN)-induced hepatocarcinogenesis in male F344 rats. Animals were given DEN dissolved in drinking water at a concentration of 40 p.p.m. for 5 weeks and then provided with drinking water free of DEN for 15 weeks to induce hepatocellular neoplasms. NIK-333 was administered orally (once a day) to rats at doses of 10, 40 and 80 mg/kg body wt for 14 weeks, starting 1 week after the completion of administration of DEN. At 20 weeks after the start of DEN administration, histopathological evaluation was carried out on all animals. The effects of NIK-333 on the cell proliferation activity of non-tumorous areas and liver tumor cells and the immunohistochemical expression of transforming growth factor-α (TGF-α) were also evaluated. NIK-333 at 40 and 80 mg/kg body wt significantly inhibited hepatocarcinogenesis ($P < 0.05$). In addition, NIK-333 at the same doses decreased DEN-induced overexpression of TGF-α in hepatocellular neoplasms (adenomas and carcinomas) and their surrounding tissue. Furthermore, NIK-333 significantly inhibited cell proliferation activity in the lesions and in non-tumorous areas ($P < 0.01$). Our results suggest that NIK-333 inhibits DEN-induced hepatocarcinogenesis through suppression of TGF-α expression and cell proliferation.

Introduction

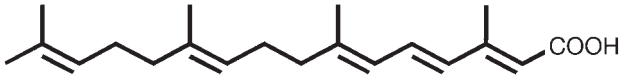
Hepatocellular carcinoma (HCC) develops through chronic hepatitis/hepatic cirrhosis caused by persistent infection with hepatitis viruses C (HCV) and B (HBV). Considerable advances have recently been made in various treatments for HCC, but high recurrence rates are still observed in patients with this malignancy and hence it still has a poor prognosis. Accordingly, inhibition of hepatocarcinogenesis at the chronic hepatitis/liver cirrhosis stage and inhibition of recurrence of HCC after treatment are important aspects of the approach to this disease. Our previous clinical trial (1) demonstrated that 1 year of treatment with an acyclic retinoid (the same material as NIK-333) significantly inhibited the recurrence of HCC following radical treatment. Moreover, NIK-333 is a drug with a high safety profile that does not cause impaired liver function or any other of the side-effects induced by other retinoids.

Since experimental studies showed that the mechanisms of inhibition of the recurrence of HCC by NIK-333 comprise induction of differentiation (2) and induction of cell death (apoptosis) (3) of liver cancer cells, we assume that these two actions of the drug work cooperatively to delete clones of latent carcinoma cells existing in hepatic tissues with chronic hepatitis/cirrhosis after radical treatment for HCC (4). Since no clear-cut comprehension of mechanisms of inhibition of carcinogenesis has, however, been achieved in animal experiments, elucidation of these mechanisms is urgently required.

The growth factor transforming growth factor-α (TGF-α) is closely involved in hepatocarcinogenesis and transformation in humans (5–9) and animals (10–13). Furthermore, the level of expression of TGF-α in liver is associated with the severity of inflammation in viral hepatitis (8,14) and elevated expression of TGF-α in hepatic cirrhosis caused by hepatitis virus is related to replication of the virus (15). Indeed, TGF-α expression is higher in liver tissue of patients with chronic hepatic diseases induced by infection with HBV or HCV as compared with normal healthy subjects (8,9,15,16). High expression of TGF-α is observed not only in carcinoma tissue but also in non-lesion hepatic tissues surrounding the carcinoma (8,9). Consequently, TGF-α is considered to play an important role in the progression from infection with hepatitis virus through liver cirrhosis to development of HCC. Experimentally, transformation of rat hepatocytes is associated with an increase in expression of TGF-α (17) and a high level of TGF-α expression is achieved in hepatic cells transformed by chemical hepatic carcinogens or hepatitis virus (18–20). Furthermore, in addition to the fact that a high incidence of HCC was observed in transgenic mice in which TGF-α was overexpressed (10,11), a dramatic increase in HCC was induced in such animals by treatment with chemical carcinogens or by overexpression of oncogenes, including simian virus 40 T antigens and mouse c-*myc* (21,22). Thus, a novel strategy for treatment of HCC by targeting TGF-α has been proposed (12,23,24).

The chemical hepatocarcinogen *N*-diethylnitrosamine (DEN) is known to induce overexpression of TGF-α in normal rat hepatocytes (12) and this phenomenon is thought to play an important role in the process of DEN-induced hepatocarcinogenesis. In the present study we have assessed the inhibitory effects of NIK-333 on hepatocarcinogenesis using a DEN-induced rat hepatocarcinogenesis model. We also investigated the effect of NIK-333 on TGF-α expression as well as its effect in modifying cell proliferation activity in hepatocellular tumors and non-tumorous liver areas.

Abbreviations: AgNORs, argyrophilic nucleolar organizer regions; DEN, *N*-diethylnitrosamine; HBV, hepatitis virus B; HCC, hepatocellular carcinoma; HCV, hepatitis virus C; RA, retinoic acid; TGF-α, transforming growth factor-α.



(2*E*, 4*E*, 6*E*, 10*E*)-3, 7, 11, 15-tetramethyl-
2, 4, 6, 10, 14-hexadecapentaenoic acid

Fig. 1. Chemical structure of NIK-333.

Materials and methods

Chemicals

The acyclic retinoid NIK-333 [(2*E*,4*E*,6*E*,*10E*)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, $C_{20}H_{30}O_2$, molecular weight 302.46, Figure 1] was synthesized by Nikken Chemicals Co. (Saitama, Japan).

Animals and treatments

A total of 120 Fischer strain male rats aged 6 weeks (F344/N SLC; Japan SLC Inc., Shizuoka, Japan) were divided into experimental groups, each consisting of 30 animals. As in our previous reports (25,26), animals were given drinking water containing 40 p.p.m. DEN (lot no. 105H0008; Tokyo Kasei Kogyo Co., Tokyo, Japan) for 5 weeks and then provided with drinking water without DEN for 15 weeks to produce liver cell neoplasms. NIK-333 (at doses of 10, 40 and 80 mg/kg body wt) or vehicle (soybean oil) was administered orally with a stomach tube in a volume of 0.5 ml/100 g body wt for 14 weeks from 1 week after completion of administration of DEN up to week 20, when the final necropsy was performed.

Daily inspection of general symptoms and weekly determination of body weight of animals were carried out during the experimental period. All surviving rats were killed by laparotomy and exsanguination from the abdominal aorta under anesthesia with sodium pentobarbital (40 mg/kg i.p.) 14 weeks after the start of administration of the test liquids. Livers were then removed, weighed, fixed in 10% neutral buffered formalin and embedded in paraffin.

Histopathology and morphometric analysis

Livers were stained with hematoxylin and eosin and with anti-TGF-α antibody for immunohistochemical examination under a light microscope. Furthermore, staining of argyrophilic nucleolar organizer regions (AgNORs) was performed to estimate the area of AgNORs per nucleus with the use of a video image processor (IPAP; Sumika Technos Co., Osaka, Japan).

TGF-α immunohistochemistry

The method used for immunohistochemical staining of TGF-α was described previously (27). Deparaffinized and rehydrated tissue sections were digested with 0.1% trypsin in phosphate-buffered saline (PBS), pH 7.2, for 15 min at 37°C. Sections were treated for 5 min with 3% H_2O_2 dissolved in methanol in order to inactivate endogenous peroxidases. After rinsing in PBS, sections were subjected to 3% normal horse serum in PBS for 20 min to block non-specific binding sites. Sections were incubated overnight with antibody against TGF-α (1:500, 4°C) (mouse monoclonal IgG_{2a}; Oncogene Research Products, Boston, MA). Sections were stained for TGF-α by the avidin–biotin peroxidase complex (ABC) method, using a mouse Vectastain Elite ABC kit (Vector Laboratories Inc., Burlingame, CA). Each slide was evaluated for the intensity and percentage of cells stained for TGF-α on scales of 0 to 3+. The intensity of stainability was scored as: 0, negative; 1+, weakly positive; 2+, moderately positive; 3+, strongly positive. The percentage of stained cells in the lesions or areas were scored as: 0, 0%; 1+, 1–5%; 2+, 6–79%; 3+, 80–100%. The data for TGF-α were expressed as the sum of these two parameters.

Statistical analysis

Comparing data from the vehicle group with those from the groups treated with NIK-333 at doses of 10, 40 and 80 mg/kg body wt, the incidences of liver cell tumors were analyzed by χ^2 test or Fisher's exact probability test, while Bartlett's test was performed for analyses of body weight, liver weight, multiplicity of liver cell tumors, number and area of liver neoplasms per unit area of specimen and area of AgNORs per nucleus. When homogeneity of variance of data was found, one-way analysis of variance was utilized, whereas the Kruskal–Wallis test was utilized in cases where homogeneity of variance of the data was not shown. If any difference among the groups was found in a test, Dunnett's multiple comparison test was performed to assess for a significant difference. The statistical significance level was set at 5%.

Table I. Body and liver weights of rats

Treatment		No. of rats	Body wt (g)	Liver wt (g)	Relative liver wt (%)
DEN	NIK-333				
+	Vehicle	30	375.1 ± 18.0[a]	12.1 ± 0.9	3.24 ± 0.20
+	10 mg/kg	30	376.0 ± 19.4	12.0 ± 0.9	3.18 ± 0.15
+	40 mg/kg	30	380.3 ± 22.4	12.2 ± 1.1	3.20 ± 0.15
+	80 mg/kg	30	361.7 ± 19.4[b]	12.4 ± 1.1	3.42 ± 0.31[b]
−	–	8	394.7 ± 43.8	12.4 ± 0.8	3.16 ± 0.35
−	Vehicle	8	368.5 ± 20.3	12.0 ± 1.2	3.09 ± 0.24
−	80 mg/kg	8	372.4 ± 17.6	11.6 ± 0.9	3.11 ± 0.18

[a]Mean ± SD.
[b]$P < 0.05$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).

Results

General observations

No animals died during the study period. There was no significant difference in body weight gain found during the period from week 0 to week 17 among the vehicle group and the groups treated with NIK-333 at doses of 10, 40 and 80 mg/kg body wt. There was also no significant difference in body weight gain found during the period from week 18 to week 20 among the vehicle group and the groups treated with NIK-333 at doses of 10 and 40 mg/kg body wt, whereas a significant decrease in body weight gain was observed in the group treated with NIK-333 at a dose of 80 mg/kg body wt as compared with the vehicle group ($P < 0.05$). Body weights, absolute liver weights and relative liver weights of rats at the end of the study are summarized in Table I. At death (week 20) there was no significant difference in absolute liver weight of each group as compared with that of the vehicle group. A significant increase in relative liver weight was observed in the group treated with NIK-333 at a dose of 80 mg/kg body wt as compared with that in the vehicle group ($P < 0.05$).

Incidence and multiplicity of hepatocellular neoplasms

The incidence and multiplicity of liver cell neoplasms at the end of the study are summarized in Table II. There was no significant difference in the combined incidence of liver cell tumors (adenoma and carcinoma) and in the incidence of hepatocellular adenomas in each group treated with NIK-333 as compared with those in the vehicle group. Decreases in the incidence of HCC were found in the groups treated with NIK-333 at doses of 40 and 80 mg/kg body wt as compared with those in the vehicle group, but these differences were not significant. As for multiplicity of hepatocellular neoplasms, there was no significant difference in the combined multiplicity of liver cell tumors (adenoma and carcinoma) or in the multiplicity of hepatocellular adenomas in each group as compared with those in the vehicle group. NIK-333 given at doses of 10, 40 and 80 mg/kg body wt lowered the multiplicity of HCCs as compared with the vehicle group, with significant differences between the groups treated with NIK-333 at doses of 40 and 80 mg/kg body wt and the vehicle group ($P < 0.05$).

Number and area of liver cell tumors per unit area

The number and area (mm^2) of liver cell tumors per unit area of specimen (cm^2) are shown in Table III. No significant difference was observed in the number of lesions per unit area of specimen (number/cm^2) with respect to total

Table II. Effects of NIK-333 on DEN-induced tumorigenesis in the liver

Treatment		No. of rats	Total tumors		Hepatocellular			
DEN	NIK-333		Incidence (%)	Multiplicity	Adenomas		Carcinomas	
					Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
+	Vehicle	30	24/30 (80.0)	2.13 ± 1.76[a]	23/30 (76.7)	1.17 ± 1.09	17/30 (56.7)	0.97 ± 0.96
+	10 mg/kg	30	25/30 (83.3)	1.90 ± 1.67	19/30 (63.3)	1.17 ± 1.21	15/30 (56.7)	0.73 ± 0.94
+	40 mg/kg	30	19/30 (63.3)	1.23 ± 1.30	18/30 (60.0)	0.87 ± 0.86	10/30 (33.3)	0.37 ± 0.56[b]
+	80 mg/kg	30	22/30 (73.3)	1.67 ± 1.88	18/30 (60.0)	1.30 ± 1.60	9/30 (30.0)	0.37 ± 0.67[b]
−	–	8	0/8 (0)	0 ± 0	0/8 (0)	0 ± 0	0/8 (0)	0 ± 0
−	Vehicle	8	0/8 (0)	0 ± 0	0/8 (0)	0 ± 0	0/8 (0)	0 ± 0
−	80 mg/kg	8	0/8 (0)	0 ± 0	0/8 (0)	0 ± 0	0/8 (0)	0 ± 0

[a]Mean ± SD.
[b]$P < 0.05$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).

Table III. Quantitative analysis of liver tumors in rats treated with DEN

Treatment		No. of rats	Total tumors		Hepatocellular			
DEN	NIK-333		No. (no./cm^2)	Area (mm^2/cm^2)	Adenomas		Carcinomas	
					No. (no./cm^2)	Area (mm^2/cm^2)	No. (no./cm^2)	Area (mm^2/cm^2)
+	Vehicle	30	1.25 ± 1.06[a]	7.48 ± 9.79	0.71 ± 0.75	1.38 ± 1.67	0.54 ± 0.53	6.10 ± 9.43
+	10 mg/kg	30	1.09 ± 0.86	8.12 ± 9.36	0.70 ± 0.74	2.28 ± 3.02	0.39 ± 0.44	5.84 ± 9.28
+	40 mg/kg	30	0.70 ± 0.69	3.26 ± 4.52	0.50 ± 0.48	1.04 ± 1.48	0.20 ± 0.30[b]	2.22 ± 3.68
+	80 mg/kg	30	0.83 ± 0.79	10.74 ± 16.72	0.66 ± 0.73	2.83 ± 4.44	0.17 ± 0.30[c]	7.91 ± 16.48
−	–	8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
−	Vehicle	8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
−	80 mg/kg	8	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a]Mean ± SD.
[b]$P < 0.05$, compared with the DEN + vehicle control (Dunnett's multiple comparison test).
[c]$P < 0.01$, compared with the DEN + vehicle control (Dunnett's multiple comparison test).

hepatic tumors (adenoma and carcinoma) and hepatocellular adenomas in each group as compared with those in the vehicle group. NIK-333 given at doses of 10, 40 and 80 mg/kg body wt lowered the number of HCCs to below that in the vehicle group and significant differences were observed between the groups treated with NIK-333 at doses of 40 and 80 mg/kg body wt and the vehicle group ($P < 0.05$ and $P < 0.01$, respectively). As listed in Table III, there was no significant difference in the area of liver cell tumors per unit area of specimen (mm^2/cm^2) with respect to total tumors (adenoma and carcinoma), hepatocellular adenomas and HCCs in each group as compared with the vehicle group.

Immunohistochemical expression of TGF-α

The effects of NIK-333 on the expression of TGF-α in the tumorous and non-tumorous areas were assessed by immunohistochemistry of TGF-α-stained sections. Strong TGF-α expression was found in hepatocellular neoplasms and non-tumorous areas (mainly around the central veins) in the DEN and vehicle treatment group, as shown in Table IV and Figure 2A–C. Lowered TGF-α expression was observed in the tumorous areas (adenomas and HCCs) in all groups treated with NIK-333 (Figure 2E and F). Similarly, in the non-tumorous areas TGF-α expression, which was increased by treatment with DEN (Figure 2A), was markedly inhibited in the groups treated with NIK-333 at doses of 40 and 80 mg/kg body wt (Figure 2D).

Table IV. Scoring of immunoreactivity of TGF-α in hepatocellular lesions

Treatment		Non-tumorous area	Adenomas	Carcinomas
DEN	NIK-333			
+	Vehicle	1.82 ± 0.90[a] (34)	4.38 ± 0.52 (8)	4.56 ± 0.73 (9)
+	10 mg/kg	1.24 ± 0.99[b] (34)	3.64 ± 0.67[b] (11)	3.89 ± 0.60[c] (9)
+	40 mg/kg	0.06 ± 0.34[d] (34)	3.67 ± 0.82 (6)	2.83 ± 1.17 (6)
+	80 mg/kg	0.06 ± 0.35[d] (33)	3.27 ± 0.79 (11)	2.14 ± 1.77[d] (7)
−	–	0 (5)		
−	Vehicle	0 (5)		
−	80 mg/kg	0 (5)		

Numbers in parentheses are numbers of areas or lesions examined.
[a]Mean ± SD.
[b]$P < 0.02$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).
[c]$P < 0.05$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).
[d]$P < 0.001$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).
[e]$P < 0.005$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).
[f]$P < 0.01$, compared with the DEN + vehicle treatment (Dunnett's multiple comparison test).

Area of AgNORs

The area of AgNORs per nucleus in the non-leison area, hepatocellular adenomas and the HCCs are shown in Figure 3. Significant decreases in the area of AgNORs per nucleus in the non-lesion areas and in the hepatocellular adenomas were

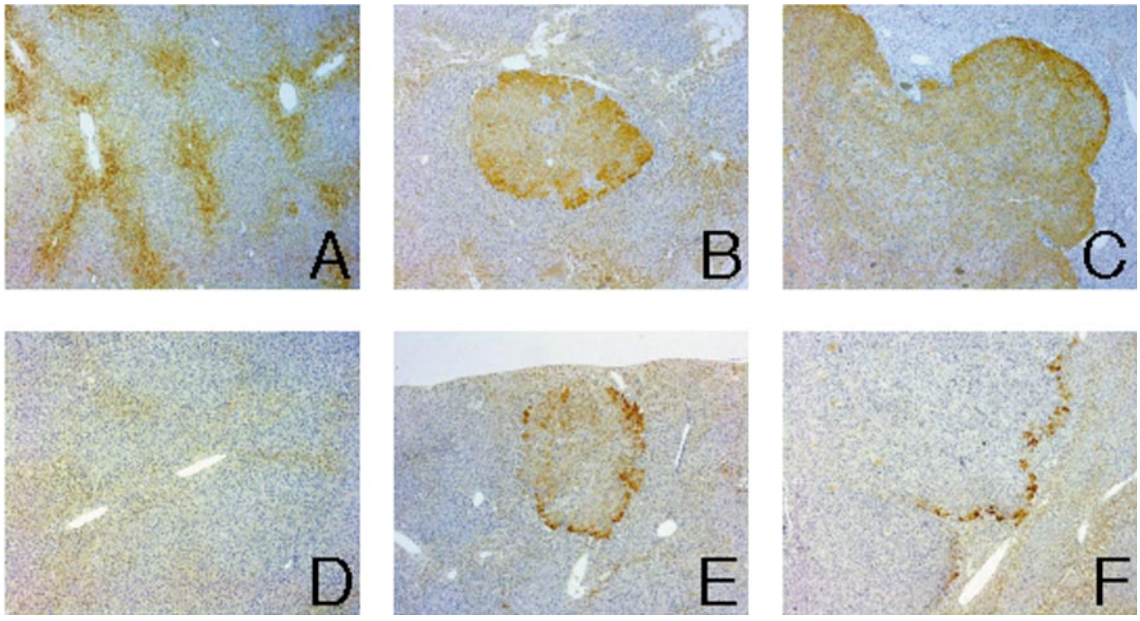


Fig. 2. Immunohistochemistry of TGF-α. (**A**) Increased expression of TGF-α in the non-tumorous area of liver from an animal after treatment with DEN (original magnification ×4). (**B**) Strong expression of TGF-α in a liver cell adenoma from a rat treated with DEN and vehicle (original magnification ×4). (**C**) Strong expression of TGF-α in a liver cell carcinoma from a rat treated with DEN and vehicle (original magnification ×4). (**D**) No expression of TGF-α in liver from a rat treated with DEN and NIK-333 at a dose of 80 mg/kg body wt (original magnification ×4). (**E**) Decreased expression of TGF-α in a liver cell adenoma from a rat treated with DEN and NIK-333 at a dose of 80 mg/kg body wt (original magnification ×4). (**F**) Markedly decreased expression of TGF-α in a liver cell carcinoma from a rat treated with DEN and NIK-333 at a dose of 80 mg/kg body wt (original magnification ×4).

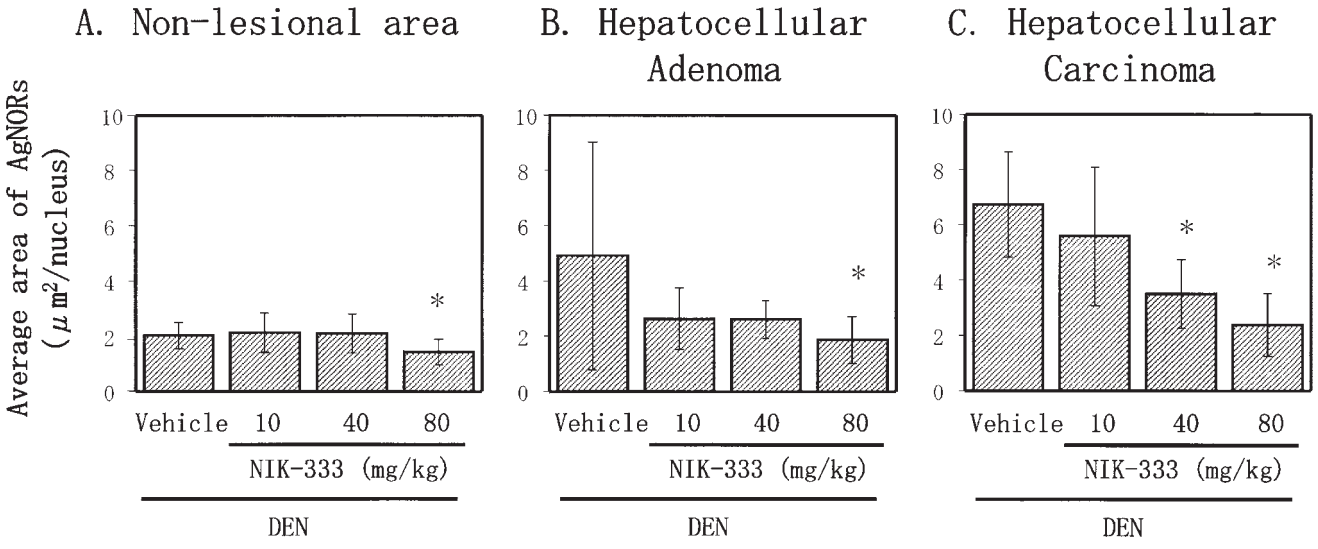


Fig. 3. Effect of NIK-333 on the area of AgNORs in DEN-treated rats (week 20). Data are means ± SD. $^{*}P < 0.01$.

found in the group treated with NIK-333 at a dose of 80 mg/kg body wt as compared with the vehicle group ($P < 0.01$). In the HCCs, NIK-333 at doses of 10, 40 and 80 mg/kg body wt reduced the area of AgNORs per nucleus in a dose-dependent manner, while significant differences in the area were observed between the groups treated with NIK-333 at doses of 40 and 80 mg/kg body wt and the vehicle group ($P < 0.01$).

Discussion

A number of retinoids have been assessed with the aim of chemoprophylaxis of chemical carcinogenesis (28,29). The mechanisms of action of such compounds include an inhibitory action on cell proliferation, an apoptosis-inducing action and a differentiation-inducing action (28–30). NIK-333, which has been developed by our research group as an agent inhibiting recurrence of HCCs, is an acyclic retinoid which shows agonistic activities against retinoic acid receptors and retinoid X receptors (2,31). In *in vitro* experiments this acyclic retinoid exerts differentiation-inducing (2) and apoptosis-inducing actions (3) in HCC cells. Thus, it is considered that these actions in collaboration block the development of HCCs (32,33).

In the present study we have assessed the effect of NIK-333 on liver TGF-α expression, which was enhanced by DEN exposure. Burr *et al*. reported that administration of DEN to

rats increased the TGF-α level in the whole liver and immunohistological staining revealed TGF-α expression in hepatocytes surrounding the centrilobular vein (12). This finding implies involvement of TGF-α in DEN-induced hepatocarcinogenesis. Although the reason why expression of TGF-α is increased in hepatocytes by treatment with DEN is not yet known, it is likely that dedifferentiation of mature hepatocytes is induced by the hepatocarcinogen. TGF-α is moderately expressed in fetal hepatocytes and plays a key role in proliferation and differentiation of hepatocytes (34). Expression of TGF-α is reduced gradually in the liver after birth (35,36) and by adulthood is negative or only slightly positive (36). Meanwhile, data proving that hepatocarcinogenesis in rats treated with DEN arises from dedifferentiation of mature hepatocytes have recently been reported (37). This may indicate that the degree of differentiation of mature hepatocytes is reduced by carcinogens. It is likely that DEN induces a decrease in the degree of differentiation of mature hepatocytes, which results in an increase in their production of TGF-α, similar to fetal hepatocytes.

The results of the present study demonstrated that DEN-induced rat hepatocarcinogenesis was inhibited by treatment with NIK-333. Furthermore, immunohistological analyses revealed that NIK-333, at the same doses as those inhibiting hepatocarcinogenesis (40 and 80 mg/kg body wt), markedly inhibited TGF-α overexpression in liver cell neoplasms, especially in carcinomas, induced by treatment with DEN. Although an acyclic retinoid (the same material as NIK-333) down-regulated cellular levels of TGF-α mRNA in established liver cancer cell lines (38), this is the first report immunohistochemically demonstrating that NIK-333 inhibits the excessive expression of TGF-α in rat liver cell tumor tissues induced by a chemical carcinogen.

It is possible that TGF-α acts as a molecule involved in malignant transformation via an autocrine or paracrine mechanism in a variety of pathways of human hepatocarcinogenesis (5–9,15). Many studies have focused on the effects of TGF-α on hepatocarcinogenesis and malignant transformation of tumors (10–13). TGF-α has been reported to be overexpressed in liver cancer tissues (9,39). TGF-α levels in patients with HCC were significantly higher than those in normal healthy subjects. Moreover, TGF-α is associated with differentiation of liver cancer cells (9,40). Experiments in rats strongly suggest the possibility that among glutathione *S*-transferase placental form-positive precancerous lesions, those which are also positive for TGF-α have higher proliferative activity and progress more rapidly to tumors as compared with those negative for TGF-α.

Several reports have demonstrated the relationship between TGF-α expression in non-lesion areas surrounding carcinomas and malignant transformation of tumors (5,41–43). On the other hand, sensitivity to TGF-α is enhanced in tumor cells. Namely, increased expression of epidermal growth factor receptor, a TGF-α-binding receptor, has been identified in various carcinoma tissues and cultured carcinoma cells, including HCC (7,42,44). Thus, tumor cells have great susceptibility to the effects not only of TGF-α produced and secreted by the tumor cells themselves (autocrine mechanism) but also to increased production of TGF-α in the tissues surrounding tumors (paracrine mechanism). In the present study, an inhibitory effect of NIK-333 was demonstrated on the expression of TGF-α in DEN-induced hepatic tumor tissues. Also, NIK-333 inhibited the overexpression of TGF-α in non-tumorous liver tissues. These results are interesting with regard to blocking carcinogenesis and malignant transformation of tumor cells. Although the mechanism of inhibition of hepatocellular expression of TGF-α in tumor areas by NIK-333 has not been elucidated, it is highly possible that altered or transformed hepatic cells induced by the carcinogen DEN (37) was normalized (redifferentiated) by a differentiation-inducing effect of the acyclic retinoid NIK-333.

Cell proliferation activity is closely related to the promotion of carcinogenesis (45). There have been reports showing that in rats vitamin A, all-*trans*-retinoic acid (RA) and 9-*cis*-RA inhibit cell proliferation in both neoplastic lesions and tissues surrounding tumors and that the anticarcinogenic effect of retinoids is based on inhibition of cell proliferation (30). Acyclic retinoids also exert an inhibitory effect on cell proliferation *in vitro* (38,46). In the present *in vivo* model NIK-333 inhibited cell proliferation activity in both the tumorous and non-tumorous areas. Meanwhile, since TGF-α is a mitogen of hepatocytes (47,48) and accelerates the hepatocyte cell cycle after the transition from G_0 to G_1 (49), inhibition of cell proliferation by NIK-333 might be associated with its inhibitory action on TGF-α expression. Involvement of TGF-α in the proliferation of hepatocytes in patients with chronic hepatic diseases has also been suggested (5,8). Accordingly, a mechanism can be invoked whereby inhibition of TGF-α expression induces a reduction in cell proliferation, which in turn results in inhibition of carcinogenesis.

In conclusion, our results strongly suggest that inhibition of TGF-α expression in liver cell neoplasms and the surrounding areas of hepatic tissues by NIK-333 is closely related to inhibition of carcinogenesis. This inhibition may lead to suppression of malignant (neoplastic) transformation. Furthermore, it is possible that the inhibitory action of NIK-333 on cell proliferation may inhibit hepatocarcinogenesis in cooperation with its differentiation-inducing (2) and apoptosis-inducing (3) actions. The compound may act at a late stage of hepatocarcinogenesis. Based on the results described here, a large scale multi-facility clinical trial of NIK-333 in subjects undergoing treatment for HCCs is now under consideration and the compound is expected to become a promising drug for inhibition of hepatocarcinogenesis and/or recurrence of liver cell carcinomas.

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