Metformin prevents liver tumorigenesis induced by high-fat diet in C57Bl/6 mice

K. Tajima,1,2 A. Nakamura,1 J. Shirakawa,1 Y. Togashi,1 K. Orime,1 K. Sato,1 H. Inoue,1 M. Kaji,1 E. Sakamoto,1 Y. Ito,1 K. Aoki,1 Y. Nagashima,3 T. Atsumi,2 and Y. Terauchi1

1Department of Endocrinology and Metabolism, 2Department of Molecular Pathology, Graduate School of Medicine, Yokohama-City University, Yokohama, Japan; and 3Department of Medicine II, Graduate School of Medicine, Hokkaido University, Sapporo, Japan

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THE PREVALENCE OF NONALCOHOLIC fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) is increasing with the growing epidemics of obesity and diabetes (32). NAFLD encompasses a clinicopathologic spectrum of disease ranging from isolated hepatic steatosis to NASH, which is a more aggressive form of fatty liver disease, to cirrhosis and, finally, hepatocellular carcinoma (HCC). The exact mechanism behind the development of HCC in NASH remains unclear; however, it has been established that hepatic steatosis is the important risk factor in the development of HCC. Metformin has recently drawn attention because of its potential antitumor effect. Here, we investigated the effects of metformin on high-fat diet (HFD)-induced liver tumorigenesis, using a mouse model of NASH and liver tumor. Metformin prevented long-term HFD-induced liver tumorigenesis in C57Bl/6 mice. Of note, metformin failed to protect against liver tumorigenesis in mice that had already begun to develop NASH. Metformin improved short-term HFD-induced fat accumulation in the liver, associated with the suppression of adipose tissue inflammation. Collectively, these results suggest that metformin may prevent liver tumorigenesis via suppression of liver fat accumulation in the early stage, before the onset of NASH, which seems to be associated with a delay in the development of inflammation of the adipose tissue.

THE PREVALENCE OF NONALCOHOLIC fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) is increasing with the growing epidemics of obesity and diabetes (32). NAFLD encompasses a clinicopathologic spectrum of disease ranging from isolated hepatic steatosis to NASH, which is a more aggressive form of fatty liver disease, to cirrhosis and, finally, hepatocellular carcinoma (HCC) (12). The exact mechanism underlying the development of HCC in association with NASH remains unclear; therefore, establishment of improved animal models that mimic human disease are important for a better understanding of the microenvironmental factors that lead to tumor progression in the liver. It has been suggested, through the use of dietary mouse models, that obesity-related hepatic steatosis might increase the susceptibility to development of malignancy in the liver (8, 41). More recently, we (25) demonstrated that long-term high-fat diet (HFD) loading, which can induce obesity, was sufficient to induce NASH and liver tumorigenesis in C57Bl/6 mice, a finding that has also been corroborated by other studies (14, 29, 41). Moreover, we also suggested that hepatic steatosis may predispose to liver tumorigenesis (25).

Metformin, a biguanide, remains the most widely used first-line drug for the treatment of type 2 diabetes (16); the drug exerts its effect predominantly by reducing hepatic glucose production (2). In addition to improving glycemic control in patients with diabetes, metformin has recently drawn attention because of its potential antitumor effect (17). Epidemiological studies have shown that metformin treatment is associated with a significantly lower risk of cancer mortality and development (7, 26, 43). Metformin may exert these effects by activating AMP-activated protein kinase (AMPK), which acts as a sensor of the cellular energy status (18). AMPK inhibits mammalian target of rapamycin (mTOR), a downstream effector of growth factor signaling, which is frequently activated in malignant cells (17). However, the precise mechanisms underlying these effects of metformin still remain unclear.

Epidemiological studies have shown that metformin significantly reduces the risk of development of liver cancer in humans (13, 26, 44). However, the mechanism underlying the antitumor effect of metformin against liver cancer remains to be fully elucidated. In the present study, we investigated the effect of metformin on HFD-induced liver tumorigenesis using C57Bl/6 male mice.

MATERIALS AND METHODS

Animals and treatments. Seven-week-old male C57Bl/6 mice were purchased from Clea Japan (Tokyo, Japan). At 8 wk of age, the mice were randomly divided into three groups: a control standard chow (SC) group, fed SC; an HFD group; and the HFD+Met group, fed the HFD and treated with metformin (250 mg/kg daily; a kind gift from Dainippon Sumitomo Pharma, Osaka, Japan). The metformin treatment, started together with the HFD, was continued for 8 or 60 wk. In the experiment involving later additional treatment, 8-wk-old C57Bl/6 mice received the HFD alone for 30 wk, followed by the HFD plus metformin treatment (250 mg/kg daily) for another 30 wk (HFD-HFD+Met group), and this group was compared with a group of mice fed the HFD alone throughout the 60 wk (HFD-HFD group). Metformin was administered in drinking water. Its concentration was adjusted weekly based on the average water consumption and body weight. The mice were housed on a 12:12-h light-dark cycle. The animals were maintained by standard animal care procedures based on the institutional guidelines. All the animal procedures were performed in accordance with the institutional animal care guidelines and the...
guidelines of the Animal Care Committee of the Yokohama City University.

Diet protocol. SC (MF; Oriental Yeast, Tokyo, Japan) and a HFD (High Fat Diet 32; Clea Japan, Tokyo, Japan) were used as described previously (25). The compositions of these diets are shown in Table 1. The fatty acid composition of the HF diet consisted of saturated fatty acid (22%; palmitic acid 12.6%, stearic acid 7.5%) and unsaturated fatty acid (77%; oleic acid 64.3% and linoleic acid 10.2%).

Measurement of biochemical parameters. Blood glucose, plasma levels of alanine aminotransferase (ALT), nonesterified fatty acids (NEFA), total cholesterol (T-Cho), LDL-Cho, and triglyceride (TG) were measured as described previously (25). The liver TG content was measured as described elsewhere (19).

Insulin tolerance test. The insulin tolerance test was performed under the fed condition as described previously (25).

Histopathological evaluation. Liver and adipose tissue samples were formalin fixed and paraffin embedded, and Masson-Goldner staining (Merck Research Laboratories, Lahway, NJ) and F4/80 antibody (Serotec, Oxford, UK) staining were performed as described previously (25, 31). All the probes were purchased from Applied Biosystems. All histopathological findings were scored by experienced pathologists who were unaware of the diets of the mice, and we surveyed liver tumors on the liver surface by macroscopic evaluation as described previously (25). The scoring system is described in detail in Table 2.

Western blot analysis. Liver protein was extracted as described previously (22). The extracts were subjected to immunoblotting with antibodies to AMPKα, p-AMPKα, p-mTOR, mTOR, p-S6K, and S6K (all from Cell signaling Technology, Danvers, MA). Densitometry was performed using Multi Gauge V3.0 software (Fuji Film Life Science, Tokyo, Japan).

RNA preparation and real-time quantitative PCR. Total RNA was prepared from portions of the liver and the epididymal fat, and cDNA synthesis and real-time quantitative PCR were performed as described previously (25, 31). All the images were acquired using a BZ-9000 microscope (Keyence).

Liver histology and scoring systems used. All histopathological findings were scored by experienced pathologists who were unaware of the diets of the mice, and we surveyed liver tumors on the liver surface by macroscopic evaluation as described previously (25). The scoring system is described in detail in Table 2.

Statistical analysis. Results are expressed as means ± SE (n). Differences between two groups were analyzed for statistical significance by Student’s t-test or Fisher exact test. Individual comparisons among more than two groups were conducted with the post hoc Fisher’s PLSD test. P < 0.05 was considered to denote statistical significance.

RESULTS

Metabolic changes observed following long-term treatment with metformin in HFD-fed C57Bl/6 mice. To evaluate the effect of metformin treatment on HFD-induced NASH and liver tumorigenesis in male C57Bl/6 mice, we performed a 60-wk study comparing SC, HFD, and HFD+Met groups. The body weight (BW) in the HFD group was higher than that in the SC group throughout the 60 wk (Fig. 1A). The BW in the HFD+Met group was significantly lower than that in the HFD group after 2 wk but comparable between the two groups from 15 wk onward (Fig. 1A). The food energy intake and random blood glucose level in the HFD + Met group were comparable to those in the HFD group throughout the 60 wk (Fig. 1, B and C). The glucose-lowering effect of insulin was impaired in the HFD group compared with that in the SC group but was restored in the HFD+Met group (Fig. 1D). There were no differences in the plasma T-Cho, LDL-Cho, TG, and NEFA levels between the HFD group and HFD+Met group (Fig. 1, E and F). The HFD group showed significantly higher liver weights and plasma ALT levels than the SC group, whereas the values of these parameters in the HFD+Met group were significantly decreased compared with the levels in the HFD group (Fig. 1, G and H). The TG content in the liver was not increased in the HFD group compared with that in the SC group, and metformin treatment had no effect (Fig. 1I).

Effects of treatment with metformin on the risk of occurrence of NASH and liver tumorigenesis in HFD-fed C57Bl/6 mice. Livers from the HFD group were severely enlarged compared with those in the SC group, whereas the livers from the HFD+Met group were only mildly enlarged (Fig. 2A). Although the SC group showed normal liver histology, the HFD and HFD+Met groups exhibited the typical features of NASH (Fig. 2, B and C) in various stages of development in the liver, including portal inflammation and blue wave-like bands of fibrotic tissue in the portal lesions. Scoring of the pathological findings showed a significant increase in the scores for liver steatosis, inflammation, and fibrosis in the HFD group compared with the scores in the SC group (Fig. 2D). The scores for inflammation and fibrosis, but not for steatosis,
were significantly improved in the HFD/H11001Met group compared with those in the HFD group (Fig. 2D). Moreover, tumors of various diameters were frequently observed on the liver surface in the HFD and HFD/H11001Met groups (Fig. 2E). Pathologically, these tumors were dysplastic nodules of two different types: either solid and nonfatty (Fig. 2, F and G) or containing large droplets of lipids (Fig. 2, H and I), or adenomas (Fig. 2, J and K). The tumor-carrying mice were observed in 68.8% (11 of 16 mice) in the HFD group but in none of the cases in the SC group. Metformin treatment significantly decreased the proportion of tumor-carrying mice [29.4 (5 of 17 mice) vs. 68.8%, P < 0.05, **P < 0.01 vs. SC; †P < 0.05, ††P < 0.01 vs. HFD+Met].

Fig. 1. Metabolic changes observed following long-term treatment with metformin in C57Bl/6 mice. A–C: changes in body weight (A), food intake (B), and random blood glucose levels (C). D: insulin tolerance test (OGTT). AUC for the glucose excursions was calculated during the OGTT from 0 to 120 min (n = 7–9). E–I: plasma lipid concentrations (E), plasma NEFA (F), ratio of liver weight to body weight (G), plasma alanine aminotransferase level (H), and triglyceride (TG) content in the liver (I). Standard chow (SC), open circles and bars; high-fat diet (HFD), filled circles and bars; HFD+Met (metformin), open triangles and left-hatched bars. Values are means ± SE; n = 16–17. *P < 0.05, **P < 0.01 vs. SC; †P < 0.05, ††P < 0.01 vs. HFD+Met.

We examined the effects of long-term treatment with metformin on the phosphorylation levels of AMPK, mTOR, and S6K in the nontumor area of the livers. The HFD+Met group showed higher phosphorylation levels of AMPK than the HFD group; however, metformin did not affect the phosphorylation levels of mTOR and S6K in the HFD-fed mice (Fig. 3A). Next, we checked the gene expression levels in the nontumor area of the livers. The expression levels of the genes encoding inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), and the NADPH oxidase complex were significantly increased in the HFD group compared with the levels in the SC group; however, metformin did not affect the expression levels (Fig. 3B). Metformin did not affect the expression level of the genes encoding lipogenic enzymes, such as fatty acid synthase (FAS) and stearoyl-CoA desaturase-1 (SCD1) (Fig. 3B). In addition, the expression levels of the genes encoding cyclin D1 and apoptosis-related genes, such as caspase-3, Bcl-2, Bax, and CHOP, were not significantly changed by metformin (Fig. 3B). These results indicate that treatment with metformin produced partial improvement in long-term HFD-induced NASH and prevented liver tumorigenesis in the C57Bl/6 mice. Moreover, our results suggested that the antitumor effect of metformin was independent of the mTOR/S6K pathway.
Effects of treatment with metformin on the risk of occurrence of nonalcoholic steatohepatitis (NASH) and liver tumorigenesis in C57Bl/6 mice. To investigate whether metformin prevented the development of liver tumors in a mouse model of NAFLD, we compared the findings in the HFD-HFD and HFD-HFD+Met groups (Fig. 4A). In our previous study, we demonstrated that 30-wk HFD loading was sufficient to induce NAFLD in C57Bl/6 mice (25). There were no differences in BW gain (Fig. 4B) or

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**Fig. 2.** Effects of long-term treatment with metformin on the risk of occurrence of nonalcoholic steatohepatitis (NASH) and liver tumorigenesis in C57Bl/6 mice. A: macroscopic findings after 60 wk. B and C: H&E (B) and Masson’s trichrome staining (C) of representative liver sections. D: NASH/NAFLD Clinical Research Network scoring system and the scores. E–K: macroscopic findings (E) and histopathological findings of sections of liver tumors stained with H&E from HFD and HFD+Met groups (F–K). Histological analysis of liver sections revealed 2 different types of dysplastic nodules, namely, solid and nonfatty (F, G) or containing large droplets of lipids (H, I), or adenoma (J, K). L and M: tumor size (L) and multiplicity (M) in livers. SC, open bars; HFD, filled bars; HFD+Met, left-hatched bars. Values are means ± SE. *P < 0.05, **P < 0.01.
random blood glucose levels (Fig. 4C) or the glucose-lowering effect of insulin (Fig. 4D) between the two groups. Metformin treatment significantly decreased the serum NEFA level (Fig. 4E), while having no effect on the plasma T-Chol, LDL-Chol, or TG levels (Fig. 4F). The wet weights of the epididymal fat pads were comparable between the two groups (Fig. 4G). The liver weight, plasma ALT level, and TG content in the liver were also comparable between the two groups (Fig. 4H–J).
Fig. 4. Metabolic changes observed following long-term treatment with metformin and risk of liver tumorigenesis in a mouse model of nonalcoholic fatty liver disease (NAFLD). A: experimental protocol. B–H: changes in body weight (B), random blood glucose levels (C), insulin tolerance test (D), plasma NEFA (E), plasma lipid concentrations (F), ratio of epididymal fat pad weight to body weight (G), ratio of liver weight to body weight (H), plasma ALT (I), and TG content in the livers (J) (n = 4–7). K and L: histopathological findings in livers from the 2 groups of mice stained with H&E (K) and Masson’s trichrome (L). M and N: tumor size (M) and multiplicity (N) in livers. HFD-HFD, filled circles and bars; HFD-HFD + Met, open diamonds and vertical striped bar. Values are means ± SE. *P < 0.05, **P < 0.01.
The two groups exhibited the typical histopathological features of NASH in the liver (Fig. 4, K and L). Moreover, metformin treatment failed to decrease the proportion of the tumor-carrying mice [83.3%, (5 of 6 mice) vs. 75% (3 of 4 mice)] or the size or number of tumors in liver (Fig. 4, M and N). These findings suggest that metformin treatment was insufficient to protect against HFD-induced liver tumorigenesis in mice that had already begun to develop NAFLD.

**Effects of short-term treatment with metformin on liver fat accumulation in HFD-fed C57Bl/6 mice.** To evaluate the effect of short-term treatment with metformin on HFD-induced liver fat accumulation, we performed an 8-wk study comparing C57Bl/6 mice fed SC, HFD, or HFD+Met. Metformin treatment significantly lost BW throughout the 8 wk (Fig. 5A), but did not affect random blood glucose levels (Fig. 5B) or glucose-lowering effect of insulin (Fig. 5C). The plasma T-Cho and LDL-Cho levels, but not the TG levels and NEFA level, were lower in the HFD+Met group than those in the HFD group (Fig. 5, D and E). Liver weights were comparable among the three groups (Fig. 5F). The HFD group showed significantly higher plasma ALT levels and TG content of the liver compared with the SC group, and metformin treatment significantly decreased the values of these parameters (Fig. 5, G and H). Livers from the HFD and HFD+Met groups were not enlarged compared with those in the SC group (Fig. 5I). The HFD group exhibited findings of...
steatosis, whereas hepatic steatosis was improved in the HFD+Met group (Fig. 5J).

We next examined the effects of short-term treatment with metformin on the phosphorylation levels of AMPK, mTOR, and S6K in the livers. Metformin did not affect the phosphorylation levels of mTOR and S6K in the HFD-fed mice (Fig. 6A). Moreover, the expression levels of the genes encoding inflammatory cytokines and lipogenic and β-oxidation-related enzymes were comparable between the HFD and HFD+Met groups (Fig. 6B). These results taken together, metformin treatment improved fat accumulation in the liver without changing the expression levels of the genes encoding lipogenic and β-oxidation-related enzymes.

Effects of short- and long-term treatment with metformin on adipose tissue functions in HFD-fed C57Bl/6 mice. We next examined the effects of short-term treatment with metformin on adipose tissue function in the HFD-fed C57Bl/6 mice. F4/80 immunostaining indicated increased crown-like structures (CLSs) in the HFD group, which was scarcely observed in either the SC or the HFD+Met group (Fig. 7A). Metformin treatment significantly decreased the weight and the mean adipocyte size of the epididymal fat pad (Fig. 7, C and D) and the mRNA expression levels of F4/80 and CD11c (Fig. 7G). The mRNA expression levels of MCP-1, TNF-α, and PAI-1 tended to be decreased by metformin, although metformin did not affect the expression level of IL-6 (Fig. 7G).

We then checked the effects of long-term (60 wk) metformin treatment on adipose tissue function. F4/80 immunostaining showed a similar degree of increase of the CLSs in the HFD and HFD+Met groups (Fig. 7B). The weight of the epididymal fat pad was significantly higher in the HFD+Met group than in the HFD group (Fig. 7E), whereas the mean adipocyte size was comparable between these groups (Fig. 7F). Metformin treatment did not affect the mRNA expression levels of F4/80, CD11c, MCP-1, TNF-α, PAI-1, and IL-6 (Fig. 7H). These results indicate that short-term metformin treatment suppressed adipocyte hypertrophy and improved HFD-induced adipose tissue inflammation, whereas long-term metformin treatment failed to improve the inflammation.

Weights of epididymal adipose tissue and liver in mice with or without liver tumors. To evaluate the relationship between the final adipose tissue mass and the incidence of liver tumors, we next checked the weights of the epididymal adipose tissue and liver in mice with or without liver tumors in the HFD and HFD-Met groups. The weight of the epididymal adipose tissue was significantly decreased in the tumor-bearing mice in the HF and HFD+Met groups compared with that in the non-tumor-bearing mice (Fig. 8A). In contrast, the liver weight was significantly increased in the tumor-bearing mice in the HF and HFD+Met groups compared with that in the non-tumor-bearing mice (Fig. 8B).

Effects of long-term treatment with a low dose of metformin on the risk of occurrence of liver tumorigenesis in C57Bl/6 mice. To evaluate the effect of long-term treatment with a low dose of metformin on HFD-induced liver tumorigenesis, we performed a 60-wk study comparing C57Bl/6 mice fed HFD,
HFD with 50 mg/kg metformin treatment, and HFD with 150 mg/kg metformin treatment. The tumor-carrying mice were observed in 75.0% (3 of 4 mice) in the mice fed HFD, 57.1% (4 of 7 mice) in the mice fed HFD with 50 mg/kg metformin, and 25.0% (2 of 8 mice) in the mice fed HFD with 150 mg/kg metformin. Metformin used at doses of 50 and 150 mg/kg dose-dependently reduced the incidence of liver tumors, although the difference in the result was not statistically significant.

**DISCUSSION**

NAFLD may progress to NASH with fibrosis, cirrhosis, and HCC (6, 38). It has been established that hepatic steatosis is an important risk factor for the development of HCC in preclinical and clinical study (25, 27, 30, 32, 41). We previously showed that a long-term HFD loading experiment reflected the natural course of progression from NAFLD to NASH to liver tumor (25). Here, we showed that metformin prevented liver tumorigenesis induced by HFD in mice. The finding was consistent with the previous study, demonstrating that metformin protected mice against chemically induced liver tumors by inhibiting pathways driving hepatic lipogenesis (4). The differences in the mechanism underlying the antitumor effect of metformin may reflect variation in the experiment model. In this study, we showed that metformin suppressed HFD-induced fat accumulation in the liver after 8 wk of treatment and improved the inflammation in the liver after 60 wk of treatment. The findings may lead to the suggestion that metformin prevents liver tumorigenesis through suppression of the natural course of pathological progression of NASH in mice.

We also indicated that metformin suppressed fat accumulation in the liver, which seemed to be associated with improvement of inflammation of the adipose tissue. Owing to the strong link between NAFLD and obesity, it is suspected that adipose tissue may have an important role in the pathogenesis of NAFLD. Indeed, there is growing evidence to implicate...
proteins secreted from adipose tissue in the onset of NAFLD (39). Feeding a HFD to mice at least initially led to a homeostatic remodeling that promoted adipose tissue expansion in response to the energy surfeit (34). By contrast, in the later stages during chronic HF feeding, adipose tissue remodeling was observed, which increased adipocyte cell death and reduced adipocyte size and loss of adipose tissue mass (34). Under the latter condition, adipose tissue cannot fully meet the demand for additional fat storage, and the adipose tissue dysfunction could contribute to lipid overflow to other organs such as liver (36, 40, 42). A recent study showed a strong link between inflammatory and morphological changes in adipose tissue and progression of steatosis to NASH (10). The overproduction of fatty acids in adipose tissues that flow to the liver via the NEFA pool is the most likely explanation for excess TG accumulation in NAFLD (9). Especially in the fasted state, adipose tissue contributed ∼80% of fatty acid content to the plasma NEFA pool (9). In the present study, metformin initially suppressed adipocyte hypertrophy and inflammation and improved fat accumulation in the liver without changing the expression levels of lipogenic and β-oxidation-related genes. These results suggest that metformin may suppress the overproduction of fatty acids in adipose tissues that flow to the liver, which could contribute to the prevention of hepatic steatosis in HFD-fed mice. However, to elucidate the contribution of NEFAs to TG accumulation in the liver, we should clarify the actual rate of NEFA uptake in the liver. In addition, it was unexpected that metformin had absolutely no effect on any parameters if administered to mice already fed a HFD for 30 wk. It is possible that the effect of metformin on the prevention of the ectopic fat deposition, could contribute to ameliorate insulin resistance in liver and skeletal muscle.

On the basis of the results of the present study, we hypothesized that the antitumor effect of metformin against HFD-induced liver tumorigenesis was mainly associated with the delay in the development of inflammation of the adipose tissue. A previous study revealed that visceral fat accumulation is an independent risk factor for HCC recurrence after curative treatment in patients with suspected NASH (28). Our data, demonstrating that metformin failed to protect against liver tumorigenesis in mice that had begun to develop NAFLD and inflammation of the adipose tissue, may lend support to the above-mentioned hypothesis. Moreover, our long-term study showed that the weight of the epididymal adipose tissue was significantly decreased in the tumor-bearing mice in the HF and HFD+Met groups compared with that in the non-tumor-bearing mice (Fig. 8A). The loss of adipose tissue mass, caused by adipose tissue dysfunction (34), may be associated with liver tumorigenesis, which may lend support to our hypothesis. However, to clarify the relevance between improved adipose tissue inflammation and liver tumorigenesis, we need to assess, using other experiment models and using other agents, exercise, or other model mice, whether the suppression of adipose tissue inflammation might directly lead to prevention of liver tumorigenesis.

Several mechanisms have been proposed to explain the direct antitumor effects of metformin. One possible mechanism could be activation by metformin of AMPK, which inhibits mTOR (17). Others include induction of cell cycle arrest through decrease by the drug of cyclin D1 expression (3) and apoptosis (37). Our results, however, showed that metformin did not affect the mTOR/S6K pathway, the expression of cyclin D1, or an apoptosis-related gene, in the liver. Therefore, further investigation is needed to elucidate the direct antitumor effect of metformin against liver tumor.

One major limitation of this preclinical study was that the dose of metformin used in this study was much higher than the dose that can be safely administered in the clinical setting. This was based on previous studies investigating the antitumor effects of metformin in mouse models, indicating the need for use of higher doses of metformin (250–300 mg/kg) in mice due to the difference in drug sensitivity between rodents and humans (5, 15, 45). On the other hand, recent studies have shown that metformin is capable of inhibiting tumor growth in mouse models even at doses equivalent to those used clinically in humans (1, 3). We showed that metformin used at doses of 50 and 150 mg/kg reduced dose dependently the incidence of liver tumors, although the difference in the result was not statistically significant.

Another limitation of the present study was the weight loss induced by metformin in the early stage of treatment. Metformin has been shown to induce body weight loss, possibly by suppressing food consumption via reducing ghrelin secretion (11, 33). However, metformin did not affect the food consumption in this study. On the other hand, a previous clinical study showed that the weight loss induced by metformin was associated with a preferential loss of adipose tissue (35). The above notion is consistent with our data; however, further study is needed to clarify whether weight loss in the early stage of treatment is directly associated with the prevention of liver tumorigenesis.

What is the relevance of the present results to the clinical management of patients with diabetes? It has been shown in preclinical (20) and single-arm clinical studies (21, 24) that treatment with metformin may ameliorate fatty liver disease in diabetic patients. However, a recent meta-analysis has indicated the lack of any effect of metformin in improving the liver histology in NAFLD/NASH patients (23). This latter finding may be consistent with the finding of our study that metformin failed to prevent liver tumorigenesis in mice that had already

![Graph](http://example.com/graph.png)

**Fig. 8.** Weights of epididymal adipose tissue and liver in mice with or without liver tumors. Experiments were performed on C57BL/6 male mice fed SC, HFD, or HFD+Met (250 mg/kg daily) for 60 wk. A: ratio of fat pad weight to body weight in non-tumor-bearing mice (NT) and tumor-bearing mice (T) of HFD and HFD+Met groups of mice: NT (n = 5), T (n = 11) in the HFD group; NT (n = 12), T (n = 5) in the HFD+Met group. B: ratio of liver weight to body weight in NT and T mice of HFD and HFD+Met groups, NT (n = 5), T (n = 11) in the HFD group; NT (n = 12), T (n = 5) in the HFD+Met group. NT, dotted bars; T, grid bars. Values are means ± SE. *P < 0.05, **P < 0.01.
began to develop NAFLD. Collectively, we propose that treatment with metformin may be useful as an early intervention, before the onset of NAFLD, to prevent liver tumorigenesis in patients with diabetes. However, further research is needed to confirm this contention.

In conclusion, metformin prevents liver tumorigenesis induced by long-term administration of a HFD in C56Bl/6 mice. This antitumor effect of metformin may be associated with a suppression of liver fat accumulation in the early stage, before the onset of NAFLD, which seemed to be associated with a delay in the development of inflammation of the adipose tissue.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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