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Molecular Mechanisms Linking Adiponectin Receptor Signalling and Cancer

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Abstract: Adiponectin is an adipose tissue-derived hormone. It is a key hormone that is responsible for insulin sensitization, and its circulating level is inversely associated with abdominal obesity. Recent studies have shown that a reduced plasma adiponectin level is significantly correlated with the risk of various kinds of cancers. Adiponectin may influence the cancer risk by modulating the metabolic environment indirectly. However several cancer cells express adiponectin receptors, suggesting that adiponectin also may modulate the cancer progression directly. Herein, we review the recent evidence concerning the molecular mechanisms linking adiponectin receptor signaling and cancer. Further studies are required to fully elucidate the molecular mechanisms of the adiponectin-mediated signaling pathway in cancer.

Keywords: Adiponectin, colorectal cancer, visceral fat, highfat diet, AMPK, mTOR.

INTRODUCTION

Adiponectin, which is also referred to as AdipoQ or ACRP30, is a 224 amino acid protein that circulates in human plasma as a homopolymer or as full-length adiponectin (fAd) that comprises 18 monomeric units. Proteolytic cleavage of full length adiponectin produces globular adiponectin (gAd), which is thought to have enhanced potency [1].

It is now well known that the adiponectin receptor exists in two isoforms; adiponectin receptor 1 (AdipoR1), which is abundantly expressed in the skeletal muscle and adiponectin receptor 2 (AdipoR2), which is predominantly expressed in the liver [1, 2].

AdipoR1 has high affinity for the globular form of adiponectin, whereas AdipoR2 exhibits intermediate affinity for both the globular and the full length adiponectin. Overexpression and gene manipulation experiments in mice have demonstrated the ability of these receptors to ligand dependently activate the 5'-AMP-activated kinase (AMPK), p38 mitogen-activated protein kinase (p38 MAPK) and peroxisome-proliferator-activated receptor-α (PPARα), as well as stimulate fatty acids oxidation and glucose uptake in murine hepatocytes [2].

PROPOSED MECHANISM UNDERLYING THE SUPPRESSIVE EFFECT OF CIRCULATING ADIPONECTIN ON VARIOUS KINDS OF CANCER

Insulin resistance and hyperinsulinemia are well-known risk factors for various kinds of cancer [3]. Adiponectin is known to be an "insulin-sensitizing hormone". Therefore, it may be possible that adiponectin improves insulin resistance resulting in an indirect suppression of cancer cell progression. In contrast, several reports, including one from our group, have demonstrated that adiponectin directly inhibits the cancer cell proliferation *in vitro* [4-6]. These results lead to the hypothesis that there may be two kinds of action of adiponectin on cancer progression and invasion, a direct effect and an indirect effect. In the direct effect on cancer, adiponectin modulates several intracellular signaling pathways via the adiponectin receptors. As noted above, there are two types of adiponectin receptors, AdipoR1 and AdipoR2, and adiponectin can stimulate AMPK, PPARα, and MAPK in classical insulin target organs such as liver and skeletal muscle [2], however it has not been fully elucidated whether these receptor-mediated signaling pathways play any roles in cancer progression.

In the indirect action of adiponectin on cancer, adiponectin may influence cancer risk by modulating the metabolic environment, such as improving insulin resistance.

INDIRECT ACTION OF ADIPONECTIN ON CANCER

Adiponectin may influence cancer risk through its well-recognized effects on insulin resistance [3]. Circulating adiponectin concentrations are inversely correlated with fasting plasma insulin [7], and adiponectin stimulates the sensitivity of peripheral tissue to insulin, which leads to a decrease in plasma insulin concentration [8]. Insulin increases bioactive insulin-like growth factor (IGF)-1 through various mechanisms. Growth hormone is the primary regulator of hepatic production of IGF-1, and the expression levels of hepatic growth hormone receptor are partly regulated by insulin [9, 10]. Recent evidence has indicated that insulin/IGF-1 is

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associated with an increased incidence or mortality rate for a number of cancers [11]. In addition to their role in metabolism, insulin and IGF axes are major determinants of proliferation and apoptosis and thus may influence carcinogenesis [12]. Previous reviews on the link between insulin/IGF-1 and colorectal cancer risk have focused on the aspects of underlying biological models [13-16], animal models [15], and some epidemiologic evidence [13, 16, 17]. *In vitro,* insulin is an important growth factor for colorectal mucosal cells and acts as a mitogen for colonic carcinoma cells [18, 19]. IGF-1 inhibits apoptosis and is required for cell cycle progression [20]. Both normal colonic epithelium and colon cancer tissues have insulin and IGF-1 receptors [21, 22]; when activated by IGF-1, the receptor-ligand complex inhibits apoptosis and allows progression through the cell cycle [18, 20, 22]. Therefore, both pre-malignant and cancerous stages of epithelium can be affected by IGF-1. It has also been reported that IGF-1 promotes proliferation and inhibits apoptosis, including that of normal prostate and tumor cells *in vitro* [23]; and elevated IGF-1 levels in most although not all studies, are associated with an increased risk of prostate cancer, particularly advanced cancer [24]. Considerable evidence suggests that the complex processes that can lead to insulin resistance are likely to play a role in pancreatic carcinogenesis [11]. Furthermore, IGF-1 and IGF-1 receptors are highly expressed in pancreatic cancer cell lines [25], and initiation of intracellular signaling through the IGF-1 receptor leads to an increase in proliferation, invasion, and expression of mediators of angiogenesis and decrease in apoptosis in pancreatic tumor cell lines [26]. The epidemiological evidence of a relationship between plasma levels of IGF-1 and risk of breast cancer has been inconsistent [27-30].

The insulin signaling pathway downstream of the insulin receptor, such as the PI3K/Akt signaling pathway, might be involved in the promotion of cell proliferation. Akt plays an important role in a variety of biological processes including cell survival, cell growth, and oncogenesis [31-33]. Therefore, the PI3K/Akt pathway is believed to play a crucial role in the development of obesity-related cancer (Fig. 1). However, *in vivo* mechanistic evidence has confirmed that this hypothesis is insufficient.

Although adiponectin decreases the plasma insulin concentration by improving the insulin resistance which may lead to inhibition of the PI3K/Akt signaling pathway and thereby resulting in reducing the cancer risk, there are several well-known insulin sensitizing and desensitizing hormones other than adiponectin such as TNFα, leptin, resistin, and free fatty acids. From a physiological viewpoint, the fact that adiponectin or other obesity-related factors do not exist *in vivo* alone but rather coexist in serum leads to the reasonable assumption that carcinogenesis may be influenced by the activation of several signaling pathways [34].

Further studies are needed to fully elucidate the indirect effect of adiponectin on cancer, especially with regard to other hormones that modulate insulin sensitivity.

PLASMA CIRCULATING ADIPONECTIN, AND ADIPONECTIN RECEPTORS IN RELATION TO CANCER

Recently, it was reported that adiponectin plays an important role in the suppression of several malignancies.

Miyoshi *et al.* conducted the case-control study and demonstrated that low adiponectin levels are significantly associated with an increased risk of breast cancer [35]. Mantzoros *et al.* also showed adiponectin is inversely associated with breast cancer risk, particularly in postmenoposal women [36]. In a prospective case-control study, Tworoger *et al.* observed no association between adiponectin and

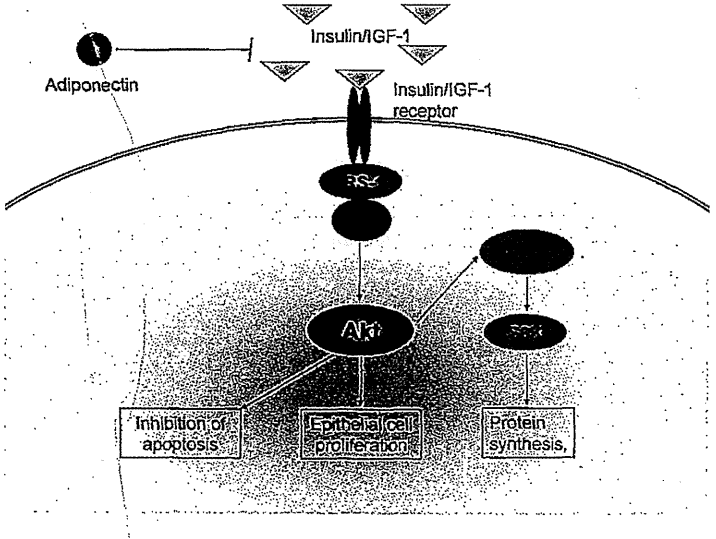

Fig. (1). Schematic illustration of indirect effect of adiponectin on cancer

Under hyperinsulinemia conditions, PI3K/Akt signaling stimulates cell growth and proliferation through many downstream substrates. Adiponectin decreases the plasma insulin concentration by improving the insulin resistance which may lead to inhibition of the PI3K/Akt signaling pathway thereby resulting in reducing the cancer risk.

breast cancer risk overall, but there was a nearly significant interaction by menopausal status (p=0.08), then they suggested that adiponectin may be inversely associated with postmenoposal breast cancer risk, particularly in a low-esterogen environment [37]. In contrast, Cust *et al.* did not observe a statistically significant inverse association between adiponectin levels and breast cancer risk in a prospective study in northern Sweden [38].

Petridou *et al.* showed that plasma adiponectin concentration is inversely and significantly related to the risk of endometrial cancer in a case-control study in Greece [39]. Dal Maso *et al.* also reported the inverse association with endometrial cancer risk emerged for plasma adiponectin levels in northeastern Italy [40]. Cust *et al.* conducted the prospective case-control study nested within the European prospective Investigation into cancer and Nutrition to examine the relation between prediagnostic plasma adiponectin levels and endometrial cancer risk, and demonstrated the negative association [41]. In prostate cancer, Goktas *et al.* showed the negative association between plasma levels of adiponectin and prostate cancer group [42]. Baillargeon *et al.* showed no association between plasma levels of adiponectin and the prostate cancer risk in a prospective study [43], whereas Sher *et al.* showed that lower adiponectin was independently associated with high-grade prostate cancer in a prospective study [44].

Kumor *et al.* reported that the serum concentration of adiponectin in colorectal carcinoma patients was lower than in controls [45], and Wei EK *et al.* showed that men with low plasma adiponectin levels had a higher risk of colorectal cancer than men with higher levels in a prospective nested case-control study [46]. In contrast, Lukanova *et al.* reported finding no association between the plasma levels of adiponectin and the risk of colorectal cancer [47], and Stocks T *et al.* did not demonstrate the negative association between colorectal cancer risk and adiponectin levels in prospective study in Sweden [48]. Therefore the association between the circulating adiponectin and colorectal cancer is controversial. Very recently, our group has demonstrated that the plasma levels of adiponectin are inversely associated with the numbers of Aberrant Crypt Foci (ACF) which is a precancerous lesion of colorectal cancer [49]. These results suggest that the adiponectin plays an important role in inhibiting colorectal carcinogenesis in its early stages.

As for the adiponectin receptors, Korner A *et al.* first demonstrated that expression of AdipoR1, but not AdipoR2, was higher in breast cancer tissue than both adjacent and control tissues [50]. On the contrary, Takahata *et al.* showed that AdipoR1 and AdipoR2 were expressed in both normal breast epithelial cells and breast cancer cells, and they also demonstrated that there was no significant difference in the mRNA expression levels of both AdipoR1 and AdipoR2 mRNA between normal breast epithelial cells and breast cancer cells [51]. Michalakis *et al.* showed that prostate cancer patients had significantly lower plasma adiponectin concentrations as compared with healthy controls. They also showed that AdipoR1 and AdipoR2 may be expressed in both prostate cancer and healthy tissue, however, weaker expressions of AdipoR1 and AdipoR2 were shown in cancerous than in healthy prostate tissue [52]. In an animal study, using azoxymethane induced chemical carcinogenesis mouse model, our group has demonstrated that a lack of adiponectin could promote colon polyp formation only under a high-fat diet condition, not under a normal diet condition, suggesting that adiponectin may play an important role in inhibiting colorectal carcinogenesis only in obese people or in those who eat a Western-style diet. Yoneda *et al.* demonstrated that AdipoR1 and AdipoR2 were expressed in normal colon epithelium and colorectal cancer tissues and that there was no significant difference in the expression of AdipoRs between normal cells and cancer cells [53]. Williams *et al*, however, reported that adiponectin receptor was expressed in normal tissue at a significantly lower level than in colorectal cancer [54].

Recently, it was reported that single nucleotide polymorphisms (SNPs) of adiponectin receptor I gene was associated with breast cancer risk [55] and colorectal cancer risk [56].

These results suggest the possibility that adiponectin might directly exert action on the suppression of cancer through the adiponectin receptors, which is different from the indirect action mediated by modulating the metabolic environment, such as improving insulin resistance. Further studies are required to elucidate the function of adiponectin and the mechanisms downstream of adiponectin receptors in malignant cells.

DIRECT ACTION OF ADIPONECTIN: ADIPONECTIN RECEPTOR SIGNALING AND CANCER

There are many reports investigating the adiponectin receptor signaling in classical insulin target organs such as liver, muscle and adipose tissues. It is reported that adiponectin stimulates AMPK, PPARα, and p38MAPK via AdipoRs in the classical insulin target organs such as liver and muscle [1, 2].

AMPK is a heterotrimetric serine-threonine kinase that senses depletion of intracellular energy and responds by stimulating catabolic pathways that generate ATP [57-61]. Under conditions in which cellular energy demands are increased or when fuel availability is decreased, intracellular ATP concentration is reduced and the AMP level rises. AMP then activates AMPK and triggers a phosphorylation cascade that regulates the activity of various downstream targets, including transcription factors, enzymes, and other regulatory proteins. One of the downstream targets of AMPK is the mTOR (mammalian target of rapamycin) pathway [57]. The phosphorylation of mTOR by AMPK plays an important role in restoring ATP levels by slowing the energy-consumption processes associated with protein synthesis and cell growth. Leptin and adiponectin are two metabolic hormones that regulate the phosphorylation state of AMPK by binding to cell surface receptors and triggering a receptor-mediated transduction cascade. Another route through which AMPK can be regulated is through activation of the tumor suppressor LKB1 [60]. The antidiabetic drugs, thiazolidinedione and metformin, also exogenously activate the AMPK [57].

Several tumor cell lines express AdipoR1 and AdipoR2, which suggests that adiponectin could exert direct effects on these cells *via* signaling through its receptors.

However, only a few reports have investigated the adiponectin receptor signaling in epithelial and cancer cells such as those of colon and stomach.

ADIPONECTIN MEDIATES mTOR/S6K SIGNALING PATHWAY IN CANCER

The mammalian target of rapamycin (mTOR), a member of the phosphoinositide 3-kinase (PI3K)-related kinase family, not only acts as a central controller of protein translation and cell cycle progression but also plays a key regulatory role for growth factors and nutritional status [62-64]. mTOR is a critical component of the PI3K and Akt pathways, and it affects cell proliferation through activation of two protein translational components, ribosomal p70S6 kinase (S6K) and eukaryotic initiation factor (eIF4E) binding proteins (4E-BPs) [65]. S6K phosphorylates the 40S ribosomal protein S6 and stimulates the translation of 5'terminal oligopyrimidine tracts mRNAs which code for ribosomal proteins and other components of the translational machinery [66]. mTOR-mediated phosphorylation of 4E-BPs dissociates the 4E–4E-BPs complex, freeing 4E for its primary function of binding to the cap structure of mRNA as part of the translation initiation complex [67]. Tuberous sclerosis complex (TSC) 1 and TSC2 are well known inhibitors of mTOR [68, 69], but AMP-activated protein kinase (AMPK) has also been reported to activate TSC1/2 [70] and directly inhibit mTOR activity [71].

In many cancers, aberrant activation of mTOR is observed. Although activating mutations in mTOR itself have not been identified, deregulations of upstream components that regulate mTOR are prevalent in cancer. Therefore, mTOR has attracted investigators' attention as a target for the molecular therapy of cancer. Clinical trials indicate that rapamycin, a specific inhibitor of mTOR, and rapamycin analogues may be effective in the treatment of multiple types of cancer [72-76].

The relationship between adiponectin and the mTOR pathway is not fully understood and their direct interaction has never been reported. In myocytes and hepatocytes, adiponectin improves insulin sensitivity and increases fatty acid oxidation through phosphorylation and activation of AMPK [77, 78]. AMPK activation by adiponectin was also observed in adipocytes, pancreatic beta cells and endothelial cells [79-82]. Caligiuri *et al.* and Wang *et al.* reported that adiponectin inhibits mTOR's downstream effectors through AMPK activation in hepatic stellate cells [83] and in myoblast cells [84], respectively. Regarding cancer cells, we reported that adiponectin decreased cell proliferation in colorectal cancer cells through activation of AMPK and inhibition of mTOR and consecutive molecules [6]. On the other hand, Barb *et al.* reported that adiponectin activated AMPK but did not inhibit mTOR in prostate cancer cells [85]. Because the effect of adiponectin on the mTOR signaling pathway may be cancer cell type specific, more detailed studies of the role of adiponectin in cancer cells are needed.

Only a few reports have been published on *in vivo* studies of the effects of adiponectin on the mTOR signaling pathway. Yamauchi *et al.* reported that treatment with adiponectin increased phosphorylation and activation of AMPK in mice liver [78]. Mariño *et al.* examined the relationship between adiponectin secretion and activity of the mTOR pathway in skeletal muscle in Zmpste24–/– mice, and found

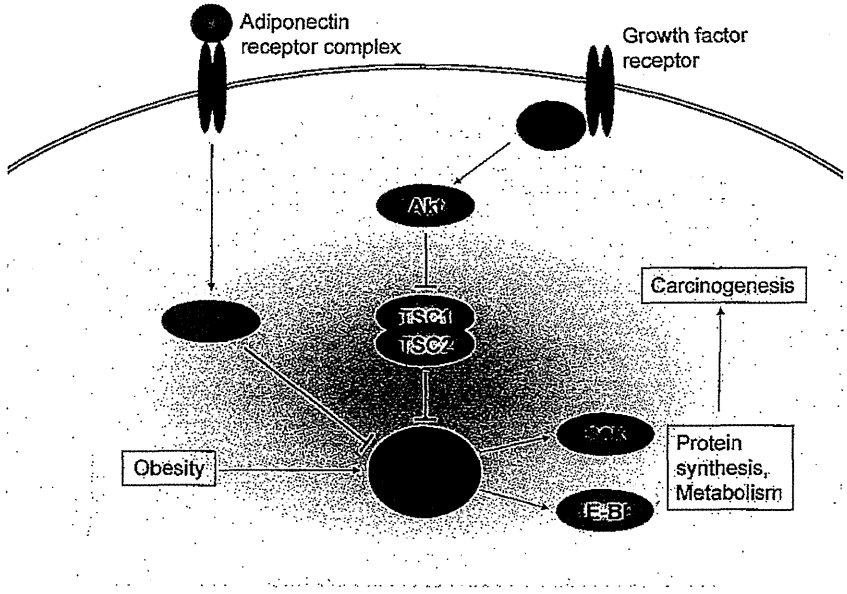

Fig. (2). Signal transduction of adiponectin in mice colon epithelium

mTOR is usually activated by PI3K/Akt pathway. Although high-fat diet induces mTOR activation, adiponectin inhibits the mTOR activation through AMPK.

that adiponectin secretion conflicted with activities of mTOR and its downstream targets [86]. We investigated the effect of adiponectin on colorectal carcinogenesis using adiponectin-deficient mice, ACRP30-/- mice [87]. Under high-fat diet conditions, adiponectin deficiency promoted proliferation of colorectal epithelial cells and their carcinogenesis but this effect was not seen under normal diet condition. Examining the activation of the mTOR signaling pathway in colorectal epithelium, AMPK phosphorylation was significantly decreased in ACRP30-/- mice and phosphorylation of mTOR, S6K, and S6 proteins were increased compared to those in wild type mice, but only under high-fat diet conditions. This activation of mTOR signaling pathway was ameliorated by replacement of adiponectin. Furthermore, administration of the specific activator of AMPK, 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) or the mTOR inhibitor, rapamycin also ameliorated the adiponectin-deficient activation of the mTOR signaling pathway. In a different study, a high-fat diet-induced obesity was associated with an increased activation of the mTOR and its downstream target S6K1 in rat liver and skeletal muscle [88]. These studies suggest that adiponectin controls the activity of mTOR elevated by obesity (Fig. 2). Activated mTOR pathway in adiponectin-deficient was associated with an increased incidence of colorectal polyps. Therefore, the control of activation of mTOR appears to be important for colorectal cancer prevention under high-fat diet conditions.

Adiponectin also increases energy metabolism *via* PPARα activation through the expression of genes involved in fatty acid uptake, intracellular transport and oxidation, as well as biosynthetic pathways. A recent report has demonstrated that PPARα may play an important role in various kinds of cancer. However no other reports have confirmed that adiponectin modulates cancer cells by activating this transcription factor.

Recently, Fenton JI *et al.* reported that adiponectin inhibited leptin-induced cell proliferation of intestinal preneoplastic cells, and that this inhibition was associated with decreased NF-κB activity [89]. The relationship between adiponectin and NF-κB is under intense investigation right now.

CONCLUSION

An indirect action of adiponectin is to improve insulin resistance, but it is difficult to clarify the effect of adiponectin in obese patients because of the low levels of circulating adiponectin in obese people. Especially in visceral type obesity, which is always associated with hyperinsulinemia, high levels of TNFα, dyslipidemia, and high levels of plasma leptin. These humoral factors interact with each other during cancer development in obese individuals, so the situation is very complicated. Therefore further studies to elucidate the role of these obesity-related humoral factors in cancer should be undertaken. We believe that the best way to clarify the effect of adiponectin in obese individuals is theoretically to administer external adiponectin to them. One of the direct actions of adiponectin in cancer development is the stimulation of the AMPK/mTOR pathway, but other adiponectin-mediated signaling pathways such as the PPARα and p38 MAPK pathways should also be investigated in various types of cancer. We think that it is a very important point that adiponectin can inhibit colon carcinogenesis and mTOR signaling pathway *via* activating AMPK only under the high-fat diet condition, not under the normal diet condition in animal study. Therefore we speculate the AMPK/mTOR signaling pathways may play an important role in obesity-related cancer. Identification of the adiponectin target molecules may in the future lead to designs of novel drugs against obesity-related cancers.

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ABBREVIATIONS

ACF	=	Aberrant crypt foci
AMPK	=	5'-AMP activated protein kinase
AOM	=	Azoxymethane
fAd	=	Full-length adiponectin
gAd	=	Globular adiponectin
IGF-1	=	Insulin like growth factor-1
mTOR	=	Mammalian target of rapamycin
S6K	=	p70 ribosomal S6 kinase

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MOLECULAR CARCINOGENESIS 49:662–671 (2010)

Metformin Suppresses Azoxymethane-Induced Colorectal Aberrant Crypt Foci by Activating AMP-Activated Protein Kinase

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Metformin is widely used for the treatment of diabetes mellitus. Adenosine monophosphate-activated protein kinase (AMPK) is known to be activated by metformin and to inhibit the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway plays an important role in the protein translational machinery and cell proliferation. We examined the effect of metformin on the suppression of colorectal carcinogenesis in chemical carcinogen-induced models. Seven-wk-old BALB/c mice were intraperitoneally (i.p.) injected with azoxymethane (AOM, 10 mg/kg) and then treated with or without metformin (250 mg/kg/d) for 6 wk (for the investigation of aberrant crypt foci [ACF] formation) or 32 wk (for polyp formation). We next investigated colonic epithelial proliferation using bromodeoxyuridine (BrdU) and the proliferating cell nuclear antigen (PCNA) labeling indices. Furthermore, to examine the indirect effect of metformin, the insulin resistance status and the serum lipid levels were assessed. Treatment with metformin significantly reduced ACF formation. The effect of metformin on colon polyp inhibition was relatively modest. No significant difference in body weight or glucose concentration was observed. The BrdU and PCNA indices decreased in mice treated with metformin. A Western blot analysis revealed that the phosphorylated mTOR, S6 kinase, and S6 protein levels in the colonic mucosa decreased significantly in mice treated with metformin. In conclusion, metformin suppresses colonic epithelial proliferation via the inhibition of the mTOR pathway through the activation of AMPK. As metformin is already used daily as an antidiabetic drug, it might be a safe and promising candidate for the chemoprevention of colorectal cancer. © 2010 Wiley-Liss, Inc.

Key words: metformin; AMPK; chemoprevention; colorectal carcinogenesis; mTOR

INTRODUCTION

Colorectal cancer (CRC) is the third most common neoplasm in developed countries [1]. Despite major advances in surgical techniques and adjuvant therapy, only a modest improvement in the survival of patients who present with advanced CRC has been achieved. One strategy for diminishing this problem is chemoprevention. Cancer chemoprevention is defined as an intervention using chemical agents that is performed before invasion or to halt or slow the carcinogenic process. Numerous studies have evaluated the possible protective effects of chemopreventive agents, such as supplemental fibers, calcium supplementation, aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), and selective cyclooxygenase (COX)-2 inhibitors [2]. The most promising agents currently available are COX-2 inhibitors [3], but recent reports have revealed an increased risk of serious cardiovascular events associated with their use [4]. The existence of these side effects led us to search for novel drugs that are both safe and effective. CRC is reportedly associated with lifestyle-related diseases such as diabetes and obesity [5–8], and these conditions might represent new targets for chemoprevention.

Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide derivative that has been widely used for a long time for the treatment of diabetes mellitus [9]. The molecular mechanism of metformin relies on LKB1-dependent activation of adenosine monophosphate-activated protein kinase (AMPK) [10].

Abbreviations: CRC, colorectal cancer; AMPK, adenosine monophosphate-activated protein kinase; APC, adenomatous polyposis coli; AOM, azoxymethane; ACF, aberrant crypt foci; TBS, Tris-buffered saline; ACs, aberrant crypts; mTOR, mammalian target of rapamycin; S6K, S6 kinase; S6P, S6 protein; BrdU, bromodeoxyuridine; PCNA, proliferating cell nuclear antigen; TUNEL, transferase deoxynucleotidyl uridine end labeling; HOMA-IR, homeostasis model assessment of insulin resistance; HPLC, high-performance liquid chromatography.

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Interestingly, patients with type 2 diabetes who are prescribed metformin have a lower risk of cancer, compared with patients who do not take metformin [11,12]. These studies suggest that metformin might be a promising candidate for the chemoprevention of CRC, at least in diabetic patients.

Nowadays, several experimental models are available for the study of colon carcinogenesis. Rodent models of colon carcinogenesis can be broadly divided into genetic models (such as $APC^{Min/+}$ mice, a murine model of familial adenomatous polyposis coli [APC]) and chemical carcinogen-induced models (such as azoxymethane [AOM]-induced models). Many chemopreventative studies have been made using a two-animal model of CRC, from which some agents show a consistent preventive effect with both models, but others have given inconsistent, conflicting results [13]. Thus, investigating whether a specific agent inhibits carcinogenesis in different animal models is important.

Previously, Tomimoto et al. [14] showed that metformin suppressed intestinal polyp growth in $APC^{Min/+}$ mice. However, the relationship between the effect of metformin and colon carcinogenesis in an AOM-induced model has not yet been investigated. The objective of this study was to reveal the effect of metformin on the suppression of colon carcinogenesis in an AOM-induced model. We studied the effect of metformin in as short-term study examining the formation of aberrant crypt foci (ACF), which are putative preneoplastic lesions of the colorectum [15,16], as well as its effect on colonic epithelial proliferation. Moreover, a long-term study of AOM-induced carcinogenesis was also conducted.

MATERIALS AND METHODS

Chemicals and Animals

AOM was purchased from Sigma (St. Louis, MO). Metformin (Sigma–Aldrich, Inc., St. Louis, MO) was mixed with the powdered basal diet Oriental MF (Oriental yeast Co., Ltd, Tokyo, Japan). The components of this basal diet were as follows: calories, 360 kcal/100 g; protein, 23.6 g/100 g; fat, 5.3 g/100 g; fiber, 2.9 g/100 g; ash, 6.1%; liquids, 7.7%. Food intake was monitored routinely on a daily basis. The mice ate the amount of about 3–4 g of the diets. The mice in the metformin-treated group were fed diets containing 2000 ppm metformin. We used metformin at a dose of about 250 mg/kg/d. The amount of metformin used in this study is higher than that used in diabetic patients (30–50 mg/kg) because previous reports investigating the antidiabetic and antitumor effects of metformin in a mouse model used a higher amount of metformin (250–350 mg/kg) since differences in drug sensitivity are known to exist between rodents and humans [17–19].

Six-wk-old BALB/c female mice were purchased from CLEA Japan (Tokyo, Japan). The mice were treated humanely according to the National Institutes of Health and AERI-BBRI Animal Care and Use Committee guidelines. All the animal experiments were approved by the Animal Care and Use Committee of the Yokohama City University School of Medicine. Four to five mice were housed per metallic cage, with sterilized softwood chips used as a bedding, in a barrier-sustained animal room air-conditioned at $24 \pm 2°C$ and 55% humidity, under a 12-h light: dark cycle. After a 1-wk period of acclimatization to the housing environment and the basal diet, the groups of mice were fed either the basal diet or the experimental diet from 7 wk of age until sacrifice.

Induction of ACF

To evaluate the effect of metformin on ACF formation, 7-wk-old mice were divided into two groups: mice fed the basal diet as a control group (Figure 1A [Group1]), or mice fed the basal diet plus metformin (Figure 1A [Group2]). Mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 2 wk and sacrificed at 6 wk after the start of AOM injection. The entire colon was removed, gently flushed with Tris-buffered saline (TBS) to remove any fecal contents, opened longitudinally, and fixed flat between filter papers in 10% neutralized formalin overnight at 4°C. After fixation, each colon was rinsed in TBS, stained with 0.2% methylene blue, and stereomicroscopically observed. ACF were identified according to previously described criteria [20,21], and the number of ACF per colon and the number of aberrant crypts (ACs) comprising each ACF were counted.

Moreover, we repeated this study to evaluate the effect of metformin on ACF formation when the metformin was administered after the completion of AOM treatment. The metformin was administered 1 wk after the final AOM injection (Figure 1A [Group3]).

Induction of Colon Polyps

Seven-wk-old mice were divided into two groups and treated with or without metformin. The mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 6 wk and sacrificed at 32 wk following the initiation of AOM injection (Figure 1B). The colon was removed and fixed in 10% neutralized formalin as described above. The number, size, and locations of the colon polyps were detected macroscopically or stereomicroscopically.

Western Blot Analysis

The colon was cut open longitudinally and washed with TBS to remove the fecal contents. Then, the colon was laid flat on a glass plate and the distal 2 cm of the colonic mucosa was scraped with a glass slide.

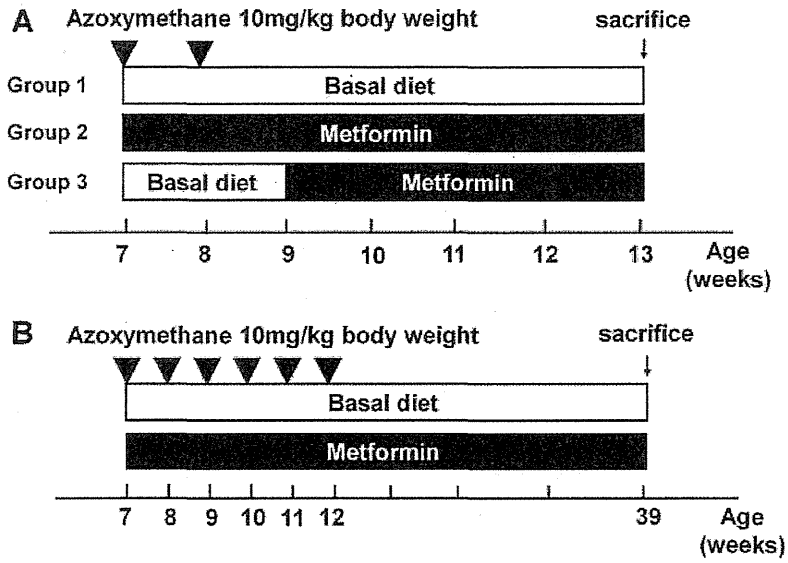

Figure 1. Experimental design for this study. (A) Protocol for AOM-induced ACF. Seven-wk-old mice were treated with or without metformin (250 mg/kg/d). Mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 2 wk and sacrificed at 6 wk after the start of the AOM injections. Group 1: Mice fed the basal diet as a control group. Group 2: Mice fed the basal diet plus metformin after the start of first AOM injection. Group 3: The metformin was administered 1 wk after the final AOM injection. (B) Protocol for the colon polyp study. Seven-wk-old mice were treated with or without metformin (250 mg/kg/d). Mice were injected intraperitoneally with 10 mg/kg of AOM once a week for 6 wk and sacrificed at 32 wk after the start of the AOM injections.

The samples were kept at −80°C until Western blot analysis. The colonic epithelial proteins were extracted using the T-PER tissue protein extraction reagent (Pierce, Rockford, IL) with 1 mM Na_3VO_4, 25 mM NaF and one tablet of proteinase inhibitor cocktail (Complete Mini; Roche, Basel, Switzerland). The protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad, Richmond, CA). The extracted protein was separated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were transferred onto a PVDF membrane (Amersham, London, UK). The membranes were blocked with 5% bovine serum albumin in TBS and probed with primary antibodies specific for AMPK, phospho-AMPK (Thr-172), mammalian target of rapamycin (mTOR), phospho-mTOR (Ser-2448), S6 kinase (S6K), phospho-S6K (Thr-389), S6 protein (S6P), phospho-S6P (Ser-235/236), Akt, phospho-Akt (Ser-473), (all from Cell Signaling Technology, Danvers, MA) and GAPDH (Trevigen, Gaithersburg, MD). Horseradish peroxidase-conjugated secondary antibodies and the ECL detection kit (Amersham) were used for the detection of specific proteins. The results were normalized to the signal generated from GAPDH.

Cell Proliferation Assay

We evaluated the bromodeoxyuridine (BrdU) and the proliferating cell nuclear antigen (PCNA) labeling indices to determine the proliferative activity of the colon epithelial cells [22–26]. BrdU (BD Biosciences, New Jersey, USA) was diluted in phosphate-buffered saline at 1 mg/mL, and 1 mg of BrdU solution was injected i.p. into each mouse 1 h prior to sacrifice. Twelve mice were tested in each group. Samples embedded in paraffin were sectioned at 4 μm thickness and stained with hematoxylin-eosin or using immunohistochemical techniques. The immunohistochemical detection of BrdU was performed using a commercial kit (BD Biosciences), and a PCNA detection kit (Zymed Laboratories, South San Francisco, CA) was used for PCNA detection. The BrdU and PCNA labeling indices were expressed as the ratio of the number of positively stained nuclei to the total number of nuclei counted in the crypts of the colon. The criteria for selecting the crypts included the presence of a clearly visible and continuous cell column on each side of the crypt. Twenty crypts were randomly evaluated in each mouse.

Apoptosis Assay

The apoptotic tumor cells were stained using a transferase deoxynucleotidyl uridine end labeling (TUNEL) staining kit according to the manufacturer's instruction (Wako Pure Chemical, Tokyo, Japan). The apoptotic index, which was expressed as the percentage of cells showing positive TUNEL staining relative to the total number of cells in the polyp, was similarly determined. Twelve mice were tested in each group.

Plasma Lipid Levels and Insulin Resistance

The levels of plasma triglycerides, cholesterol, insulin, and glucose were measured using a WAKO enzyme-linked immunosorbent assay (ELISA) kit ($n = 12$ from each group). Metformin is widely used as an antidiabetic drug to improve insulin resistance. Therefore, the status of insulin resistance, as represented by the homeostasis model assessment of insulin resistance (HOMA-IR), was investigated [27]. HOMA-IR was calculated using the following formula: HOMA-IR fasting = plasma immunoreactive insulin (IRI; μU/mL) × fasting plasma glucose (FBG; mg/dL)/405. We also measured the plasma concentration of triglycerides and cholesterol according to the manufacturer's instructions.

Influence on AOM Carcinogenicity by the Metformin Treatment

It is important to assess whether metformin affects the metabolism of AOM, since a chemical carcinogen-induced model of CRC would not be feasible if metformin inhibits the metabolism of AOM. Consequently, we assessed the carcinogenicity of AOM. The process of AOM-induced carcinogenesis is initiated by methylation of DNA after metabolism of AOM to methylazoxymethanol (MAM) by cytochrome P450 IIE1 in the liver [28] and transport to the colon via the blood stream [29]. Two major DNA adducts, N^7-methylguanine (7-MeG) and O^6-methylgunaine (O^6-MeG), have been identified in DNA of rats treated with dimethylhydrazine [30]. DNA adducts can result directly in mutational events, potentially leading to cancer. O^6-MeG in the target tissue is more closely correlated with carcinogenicity than the more frequently occurring 7-MeG [31]. We studied the effects of metformin on the formation of AOM-induced DNA adducts (O^6-MeG) using a high-performance liquid chromatography (HPLC) analysis. The experiment was performed as described previously [32]. Five mice in each group were sacrificed 6 h after the final AOM injection. The liver and colon were immediately removed and rinsed in the ice-cold saline. The colonic mucosa was scraped with a glass slide. The samples were kept at −80°C until DNA adducts analysis. Tissue DNA from the liver and colorectal mucosa was isolated with DNAZOL reagent (Invitrogen Corp., Carlsbad, CA) and precipitated with ethanol. The pellet was suspended in 0.1 M HCl (5 mg/mL) and hydrolyzed at 70°C for 30 min to release the purines as free bases. The DNA hydrolysates were analyzed by the method of Herron and Shank [31]. The procedure was performed using HPLC with a PU-980 intelligent HPLC Pump (JAPAN SPECTROSCOPIC CO., LTD, Tokyo, Japan) equipped with a Shimadzu RF-550 fluorescence monitor (Shimadzu Corp., Kyoto, Japan) at a 366-nm emission with and a 286-nm excitation. An analytic column, Whatman Partisil-10 SCX (250 × 4.5 mm; GL Sciences Inc., Tokyo, Japan), with the same type of guard column was used. The O^6-MeG was eluted with a 0.05-M ammonium phosphate solution at pH 2.5 and a flow rate of 1.0 mL/min. The results are expressed as the ratio of O^6-MeG to guanine (G) in nmol/μmol.

Statistical Analysis

Results were expressed as mean ± standard error. A statistical analysis of the blood test results was conducted using the Mann–Whitney *U*-test. Other statistical analyses were performed using the Student *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of Metformin on the Formation of ACF

To investigate the effect of metformin in suppressing colon carcinogenesis, we analyzed colon specimens for the formation of ACF as a surrogate biomarker of the early stage of colorectal carcinogenesis [15,16]. After 6 wk of AOM administration, mice fed the basal diet without metformin developed 7.0 ± 0.5 ACF per mouse; treatment with metformin significantly reduced this number to 2.2 ± 0.3 ACF. The total number of ACs per mouse was also suppressed significantly (Table 1). The administration of metformin did not significantly alter the body weight (Figure 2A) or food intake and did not cause any clinical signs of nutritional deficiency, such as diarrhea, during the 6-wk observation period.

Moreover, when the metformin was administered after the completion of AOM treatment, a significant difference in the number of ACF was observed in group 3 (Table 1). Thus, metformin can effectively inhibit ACF formation after the initiation of AOM treatment.

Table 1. Effect of Metformin on the Formation of ACF

Diet	No. of mice	No. of ACF/mouse[a]	No. of ACs/mouse[a]
Basal diet (group 1)	12	7.0 ± 0.5	11.9 ± 0.6
Metformin (group 2)	12	2.2 ± 0.3[b]	3.4 ± 0.3[b]
Metformin (group 3)	12	2.9 ± 0.4[b]	4.1 ± 0.5[b]

[a]Mean ± standard error.
[b]Differences were significant compared with the values in the control; $P < 0.05$.

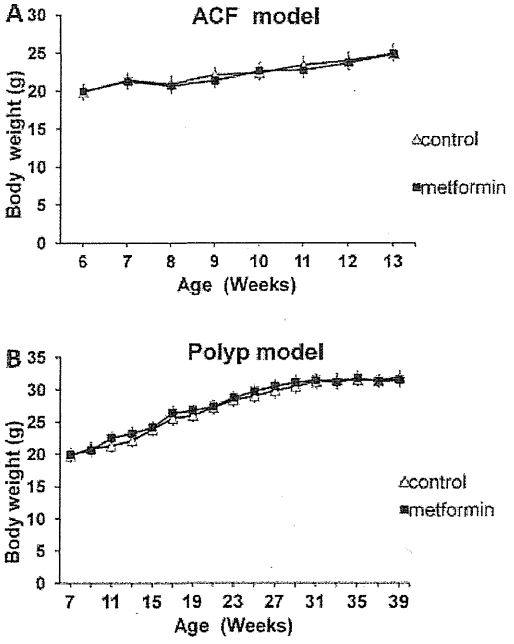

Figure 2. Mean weights of mice in the control and metformin-treated groups. (A) ACF model. (B) Polyp model. No statistically significant differences in bodyweight were observed between the metformin-treated and control groups. Data are expressed as the means ± SE.

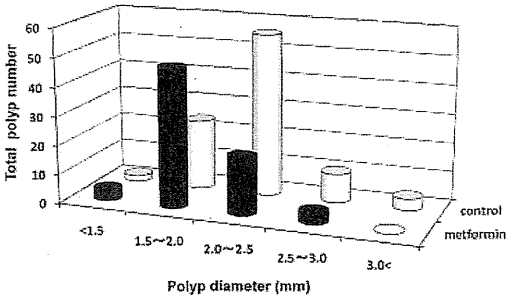

Figure 3. Size distribution of polyps. Treatment with metformin reduced the polyp size. Larger polyps, particularly those >3 mm in diameter, were not found in the metformin-treated mice.

Treatment With Metformin Inhibits Polyp Formation

To investigate the effect of metformin in polyp formation, we treated mice with metformin for 32 wk (from 7 to 39 wk of age). Treatment with metformin significantly inhibited polyp formation (number) and polyp expansion (size) in mice (Table 2). Larger polyps, particularly those >3 mm in diameter, were not found among the metformin-treated mice (Figure 3). All the animals survived the experimental period and remained in good health, with no clinical signs of nutritional deficiency. At the end of the experiment, the average body weight of the animals did not differ statistically between the experimental groups (Figure 2B).

Effect of Metformin on Plasma Lipid Levels and Insulin Resistance

Metformin is widely used as an antidiabetic drug that improves insulin resistance in patients with diabetes mellitus. Therefore, we investigated the effect of metformin on insulin resistance and the plasma lipid levels. No significant differences in the blood glucose levels and insulin resistance, as represented by the HOMA-IR, were observed between the control and the metformin-treated groups at our experimental dosages. In addition, the plasma triglyceride and cholesterol levels between the control and metformin-treated mice showed no significant difference (Figure 4).

Effects of Metformin on Colonic Epithelial Cell Proliferative Activity

To gain insights into the mechanism of metformin-induced ACF inhibition, we investigated the proliferative activity of the colon epithelium by determining the BrdU and PCNA labeling indices. Both indices were significantly lower in the metformin-treated mice than in the control group (Figure 5). These results suggest that metformin-induced inhibition of ACF formation is associated with the inhibition of cell proliferation.

Effects of Metformin on Apoptosis-Inducing Activity

To investigate whether the administration of metformin induced apoptosis, we performed TUNEL staining of the polyp tissues. The percentage of apoptotic cells was not significantly altered in the metformin-treated group, compared with in the control group (Figure 6A and B). This result suggests that metformin did not induce apoptosis.

Table 2. Effect of Metformin on the Induction of Colon Polyps

Diet	No. of mice	No. of polyps/mouse[a]	Size of polyps (mm)[a]
Basal diet (control)	12	8.0 ± 0.4	2.18 ± 0.05
Metformin	12	6.4 ± 0.4[b]	1.94 ± 0.04[b]

[a]Mean ± standard error.
[b]Differences were significant compared with the values in the control; $P < 0.05$.

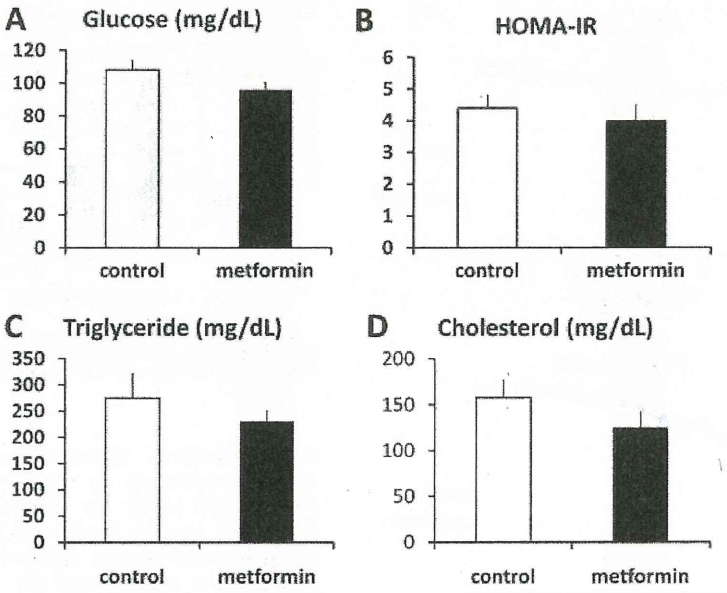

Figure 4. Plasma levels of glucose, triglyceride, cholesterol, and HOMA-IR. Mice were fed a basal diet (control) or a diet containing metformin (250 mg/kg/d). Plasma and blood samples were obtained after 12 h of fasting. (A) Blood glucose, (B) homeostasis model assessment of insulin resistance (HOMA-IR) levels, (C) triglyceride, and (D) cholesterol levels are shown. HOMA-IR was used to calculate insulin resistance using the following formula: HOMA-IR = fasting plasma immunoreactive insulin (IRI; μU/mL) × fasting plasma glucose (FBG; mg/dL)/405. No significant differences were observed in the plasma levels of glucose, cholesterol, triglyceride or HOMA-IR between the metformin-treated and control groups. Each bar represents the mean (with the SE).

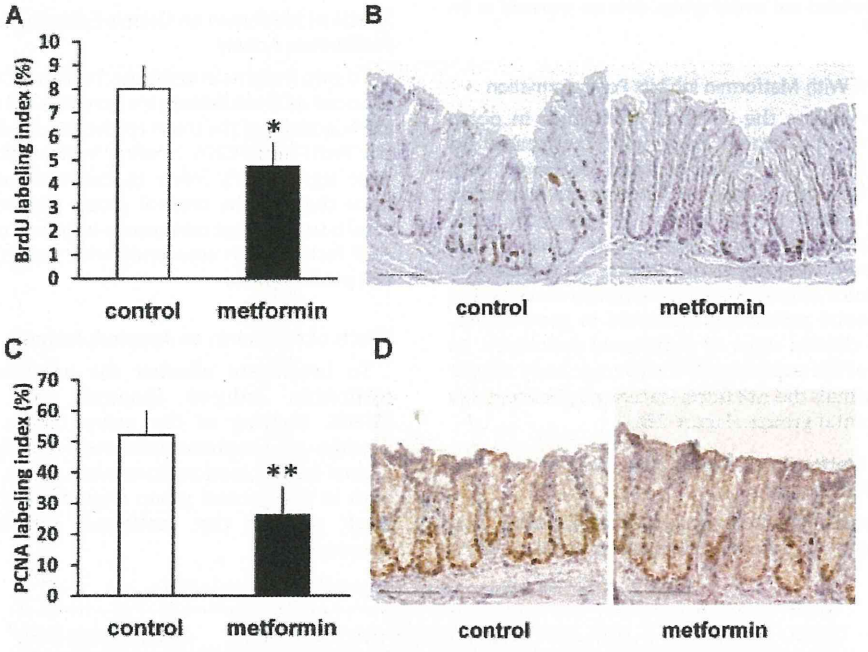

Figure 5. Cell proliferation assay using bromodeoxyuridine (BrdU) and proliferating cell nuclear antigen (PCNA). (A and C) Average BrdU labeling index (A) and PCNA labeling index (C) in each group in the ACF formation experiment. BrdU was administered intraperitoneally 1 h prior to the sacrifice of the animals. Both indices were expressed as the percentage of positively stained nuclei out of the total number of nuclei counted in the crypts of the colon. Each bar represents the mean (with the SE), $*P<0.05$, $**P<0.01$. (B and D) Representative immunohistochemical staining for BrdU (B) and PCNA (D) in each group. (Scale bars: 100 μm).

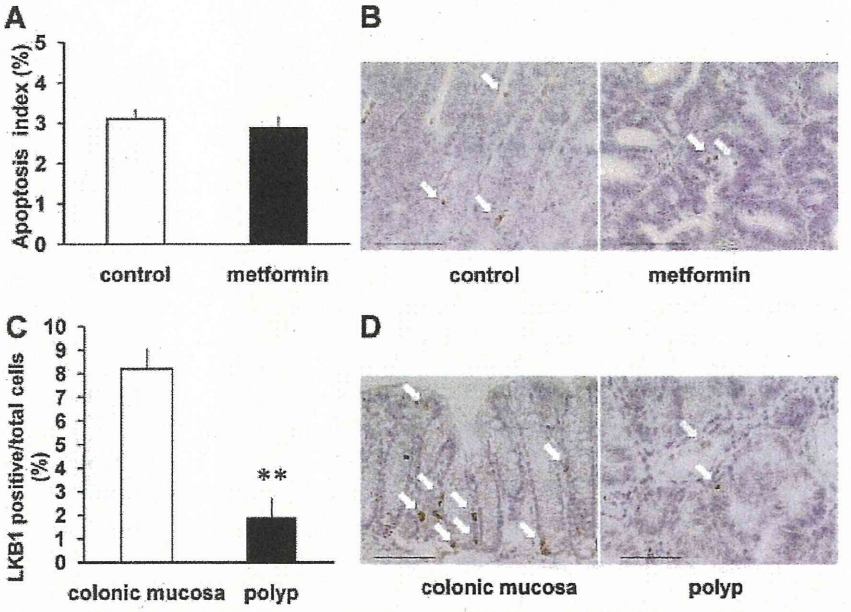

Figure 6. (A and B) Apoptosis assay using TUNEL staining. (C and D) LKB1 immunohistochemistry. (A) Average apoptosis index. (C) LKB1 positive/total cells. Both indices were expressed as the percentage of positively stained nuclei out of the total number of nuclei counted. Each bar represents the mean (with the SE), $*P<0.05$, $**P<0.01$. (B and D) Representative immunohistochemical staining for TUNEL (B) and LKB1 (D) in each group (scale bars: 100 μm).

Metformin-Mediated Activation of AMPK Inhibits the mTOR/S6K Pathway

To clarify the mechanisms responsible for the metformin-induced inhibition of cell proliferation in the colon epithelial cells, we investigated the expression levels of various potential target proteins. One of the pharmacological targets of metformin is thought to be AMPK [33], which reportedly inhibits the mTOR/S6K signaling pathway [34]. Therefore, we hypothesized that metformin could inhibit the mTOR/S6K signaling pathway through the activation of AMPK, resulting in the down regulation of the protein synthesis machinery. A Western blot analysis revealed that treatment with metformin stimulated AMPK phosphorylation (Figure 7). Furthermore, the phosphorylation of mTOR and S6K proteins was significantly inhibited by the metformin treatment. Moreover, to confirm that metformin induces the inhibition of mTOR through AMPK, we investigated whether Akt phosphorylation did not change with metformin administration. A Western blot analysis revealed that no differences in the protein levels of phosphorylated Akt were observed between the control and metformin-treated groups.

On the other hand, since the metformin-induced activation of phospho-AMPK is LKB1 dependent [10], we examined whether LKB1 existed in the colon using LKB1 immunohistochemistry (from Cell Signaling Technology). As a result, LKB1 was found to exist in both the colonic mucosa and polyps. However, the incidence of LKB1 in the polyps was lower than the colonic mucosa (Figure 6C and D). And, no significant differences in the incidence of LKB1 were observed between the control and the metformin-treated groups (data not shown).

Influence on AOM Carcinogenicity by the Metformin Treatment

No significant difference in the O^6-MeG levels was observed between the control group and the metformin-treated group in the liver and colon (Figure 8). These results revealed that metformin did not affect the carcinogenicity of AOM.

DISCUSSION

In the present study, we clearly showed the preventive effect of metformin on mouse colon carcinogenesis induced by AOM, with no toxic effects in terms of body weight loss. In a short-term experiment, metformin significantly suppressed the formation of ACF and the average size of the ACF, which was defined as the average number of ACs composing each ACF. In a long-term experiment, the effect of metformin on polyp inhibition was relatively modest.

Metformin is widely used as an antidiabetic drug that improves insulin resistance. Therefore, we investigated the effect of metformin on insulin

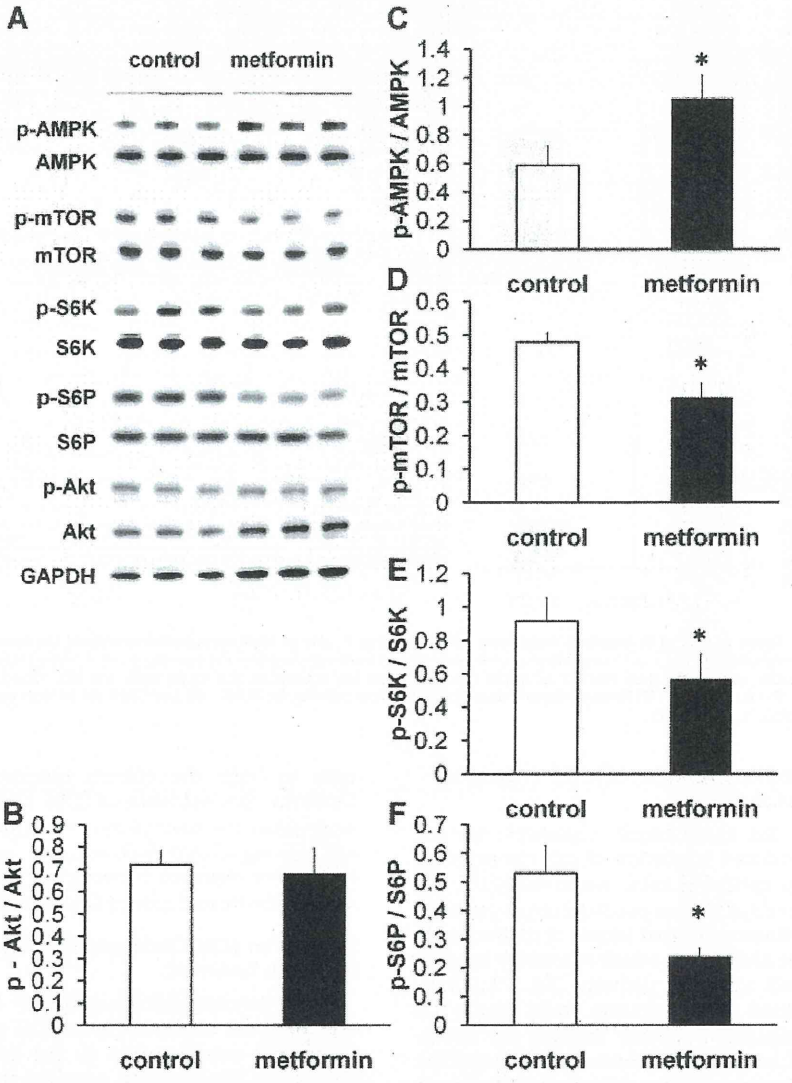

Figure 7. Treatment with metformin activates AMPK and downregulates the mTOR/S6K pathway. (A) Western blot analysis for phosphorylated and total AMP-activated protein kinase (AMPK), mTOR, S6K, S6P, and Akt in colonic mucosa from mice treated with a basal diet (control) or a diet containing metformin for 32 wk. The results were normalized to the signal generated from GAPDH. (B–F) Graphs showing the ratios of the phosphorylated protein to the total protein. Each column represents the mean (with the SE), $*P < 0.05$.

resistance and the plasma lipid levels to determine the indirect effects of this drug. Our results demonstrated that metformin did not attenuate the blood glucose, HOMA-IR, plasma triglyceride or cholesterol levels in mice, indicating that its suppressive effect on ACF formation was due to some direct effect other than the attenuation of insulin resistance or hyperlipidemia. To examine the direct effects of metformin on the suppression of ACF and polyps, we analyzed cell proliferation in the colon epithelium using BrdU and PCNA immunostaining. Both the BrdU and PCNA indices significantly decreased. In addition to these results, the apoptotic cell index was not altered by the metformin treatment. Furthermore, a Western blot analysis revealed that the metformin treatment stimulated AMPK phosphorylation, resulting in the inhibition of mTOR and other downstream components, such as S6K and S6P. These data suggest that the inhibition of the mTOR pathway by the metformin-mediated activation of AMPK leads to the suppression of cell proliferation, thereby inhibiting ACF formation. On the other

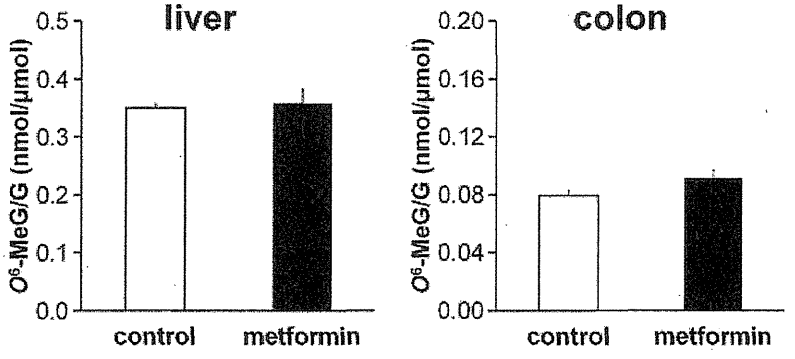

Figure 8. Effect of metformin on the formation of DNA adducts (O^6-MeG) by AOM in the liver and colon. No significant difference in the O^6-MeG levels was observed between the control group and the metformin-treated group in the liver and colon. O^6-MeG, O^6-methylguanine; G, guanine. Each bar represents the mean ± SE.

hand, the incidence of LKB1 in the polyps was lower than the colonic mucosa. So the effect of metformin on polyp inhibition might be relatively modest, because metformin-induced activation of phospho-AMPK is LKB1 dependent [10].

Inhibition of the mTOR pathway might be one direct means of suppressing ACF. We speculate that activation of the mTOR pathway directly promotes colonic epithelial cell proliferation and, consequently, colorectal carcinogenesis. The mTOR signaling pathway is known to play important roles in the protein synthesis machinery. The most characterized downstream effecter of mTOR is S6K, which regulates the initiation and elongation phases of translation [35]. The activation of the mTOR pathway accelerated the cell-cycle progression from G1 to S in DLD-1 cells [36]. Therefore, when AMPK is activated, cell growth and proliferation might be inhibited as a result of limitations in protein synthesis. Recent evidence indicates that metformin has suppressive effects on tumorigenesis and cancer cell growth [37,38]. In a human breast carcinoma cell line, metformin was able to activate AMPK, resulting in a decrease in proliferation and a general decrease in protein synthesis in vitro [37]. Metformin has also been shown to inhibit the proliferation of human prostate cancer cells [38]. Fujisawa et al. [39] demonstrated that treatment with rapamycin, an mTOR inhibitor, reduced the BrdU index and ACF formation in adiponectin-deficient mice subjected to high-fat diet conditions. Thus, AMPK activation has been demonstrated to have a potent anti-proliferative effect.

Previously, we reported that metformin suppressed intestinal polyp growth in $APC^{Min/+}$ mice [14], which is a genetic model of carcinogenesis. In the case of $APC^{Min/+}$ mice, the tumors develop mostly in the small intestine, while colon tumors are less frequent. Moreover, no typical ACF arise above the intestinal mucosa. Consequently, the progression of ACF to carcinoma cannot be established using this model. On the other hand, in chemical carcinogen-induced models, the tumors develop mainly in the colon through a multistep process similar to that observed in human carcinogenesis. In the present study, we confirmed the effect of metformin on the suppression of ACF in an AOM-induced model. This is the first report on the inhibitory effect of metformin on colorectal tumorigenesis in a chemical carcinogen-induced model. Therefore, we have confirmed the effect of metformin on tumor suppression in two different models. These results are very useful for the planning of new clinical trials, but further clinical studies are needed. For example, since the effect of metformin on polyp inhibition was relatively modest, the patient after the colonoscopic polypectomy might be a good candidate of the chemoprevention by the metformin.

In conclusion, the present study demonstrated that metformin has the potential to suppress the AOM-induced development of ACF in mice through the activation of AMPK, resulting in the inhibition of the mTOR/S6K signaling pathway and the subsequent inhibition of the protein synthesis machinery. Metformin is already used daily in humans as an antidiabetic drug, so it might be a safe and promising candidate for the chemoprevention of CRC.

ACKNOWLEDGMENTS

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Leptin receptor is involved in STAT3 activation in human colorectal adenoma

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The possible role of leptin in colorectal tumors has been investigated in previous studies; however, to date, the conclusions remain under debate. Therefore, we investigated the serum leptin levels in colorectal adenoma patients. In addition, expression of the leptin receptor, and the leptin receptor-mediated signaling pathways were investigated in biopsy specimens collected from human patients with colorectal adenoma. No significant difference in the mean serum leptin level was observed between the colorectal adenoma patients and the control subjects; however, increased expression and activation of the leptin receptor, as indicated by findings such as the phosphorylation of Tyr 1141, was observed in the colorectal adenoma tissues. In addition, activation of the JAK/STAT signaling pathway mediated by the leptin receptor and increased transcriptional regulation of downstream target molecules were observed in colorectal adenomas compared with the non-adenoma tissues. These results indicate STAT3-mediated leptin receptor signaling pathways may be activated in human colorectal adenomas. (*Cancer Sci* 2011; 102: 367–372)

Colorectal cancer is a major cause of mortality and morbidity worldwide;[1] however, the mechanism of colorectal carcinogenesis remains unclear. Recently, the existence of an association between obesity or metabolic abnormalities and an elevated risk of colorectal cancer was reported.[2,3] Adipose tissue was reported to be not only an energy storage organ, but also an active endocrine organ that secretes important adipocytokines such as adiponectin, leptin, tumor necrosis factor-α (TNF-α), free fatty acid and resistin.[4,5]

Leptin is a 167-amino acid peptide that plays a central role in the hypothalamus in relation to mammalian feeding behavior and energy expenditure.[6] Plasma leptin levels have been reported to be strongly correlated with the body mass index (BMI) in humans[7–9] and also to be elevated in obese subjects. Leptin exerts its activity thorough its specific membrane receptor, the leptin receptor (ObR), belonging to the class 1 cytokine receptor family.[5] Two isoforms, the long and short variants of ObR, namely, ObRL and ObRS, have been identified, and only the long isoform of ObR has been shown to have full signaling potential, with the short isoform showing diminished or abolished capacity for signaling.[5,10]

Several studies have reported the association between serum leptin levels and the presence of several cancers such as prostate[11–13] and breast[14,15] cancer. Similarly, previous studies have also shown an association between serum leptin levels and the presence of colorectal cancer.[16–23] However, the results of these previous studies are contradictory and difficult to interpret. While some studies have shown a decrease in the serum leptin levels in colorectal cancer patients,[16–20] others have reported elevated serum leptin levels in colorectal cancer patients.[21–23] Thus, the association between leptin and the presence of colorectal cancer has not yet been clarified. In addition, previous studies have shown that leptin stimulated cell proliferation in several types of carcinoma cell lines *in vitro*.[24–26] However, the molecular mechanisms underlying the promotion of human colorectal carcinogenesis by leptin remain unclear.

In the present study we investigated the association between plasma leptin levels/leptin receptor-mediated signaling and the development of colorectal adenoma.

Materials and Methods

Study population. One hundred and forty-four patients who underwent endoscopic mucosal resection for colorectal adenoma between June 2006 and April 2009 at Yokohama City University Hospital, and 64 control subjects who were detected to have no colorectal polyps on colonoscopy were recruited for this study. The exclusion criteria were subjects with colorectal carcinoma, familial adenomatous polyposis, inflammatory bowel disease, radiation colitis or any malignant disease, and also subjects with a previous history of colectomy, gastrectomy or colorectal polypectomy. Written informed consent was obtained from all subjects prior to their participation in the study. The study protocol was approved by the Yokohama City University Hospital Ethics Committee.

Collection and analysis of blood samples for determination of the leptin levels. Blood samples were obtained in the morning on the day of colonoscopy after the subjects had fasted overnight. Serum leptin levels were measured by enzyme-linked immunosorbent assay of human leptin (SRL Co., Tokyo, Japan).

Immunohistochemical analyses. The expressions of ObR and phospho-STAT3 (p-STAT3) were investigated in the colorectal adenoma and normal colorectal tissues. A total of 61 adenoma tissue samples were obtained endoscopically from the study subjects. Formalin-fixed and paraffin-embedded samples were deparaffinized and rehydrated. The sections were incubated with antibodies for ObR (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and p-STAT3 (Tyr 705) (1:50; Cell Signaling Technology, Danvers, MA, USA) as the primary antibodies, using an LSAB2 kit (Dako Cytomation, Carpinteria, CA, USA). They were then incubated with biotinylated immunoglobulin as the secondary antibody and treated with peroxidase-conjugated streptavidin. The antibody complex was visualized with 3,3′-diaminobenzidine, tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan). The expressions of ObR and p-STAT3 were analyzed by light microscopy in 10 different fields of each section, and the mean percentage of adenoma cells that showed positive staining was scored by two pathologists. The ObR and p-STAT3 expressions were classified into two categories depending on the percentage of cells showing positive staining:

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negative, 0–15% of all the tumor cells showing positive staining; and positive, >15% of all tumor cells showing positive staining, as previously described.[27]

Western blot analysis. Twenty-five colorectal adenoma patients were randomly selected, and biopsy samples obtained from the colorectal adenomas and normal areas were isolated. The extracted protein was separated using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, London, UK). The membranes were probed with primary antibodies specific for phospho-ObR (p-ObR) (Tyr 1141), p-ObR (Tyr 985), ObR (Santa Cruz Biotechnology), phospho-JAK2 (p-JAK2), JAK2, p-STAT3 (Tyr 705) STAT3 (Cell Signaling Technology) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Trevigen, Gaithersburg, MD, USA). Horseradish-peroxidase-conjugated secondary antibodies and the electrochemi-luminescence (ECL) detection kit (Amersham) were used for the detection of specific proteins.

Real-time RT-PCR. Twelve colorectal adenoma patients were randomly selected and biopsy samples of the adenoma and adjacent normal tissues obtained from the colorectal adenoma and normal areas were isolated. Total RNA from the colorectal adenoma and normal tissue biopsy specimens was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany). For the real-time reverse-transcriptase polymerase chain reaction, total RNA was reverse-transcribed into cDNA and amplified using the real-time quantitative polymerase chain reaction using the Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Probes and primer pairs specific for ObRL, ObRS, BclX, c-Myc, cyclin D1, cdc2, cyclin B1, VEGF and 18S were purchased from Applied Biosystems. The concentrations of the target genes were determined using the competitive computed tomography method and the values were normalized to the internal control.

Statistical analysis. Statistical analyses were performed using the Mann–Whitney *U*-test and chi-square test. All analyses were performed using the Stat View software (SAS Institute, Cary, NC, USA). $P < 0.05$ was regarded as denoting statistical significance.

Table 1. Characteristics of the study patients

	Normal	Adenoma	*P* value
N	64	144	
Age (years)	62.1 ± 13.8	64.7 ± 10.2	0.12
Sex (M/F)	33/31	100/44	0.18
Waist circumference (cm)	84.4 ± 10.3	86.5 ± 10.5	0.28
BMI (kg/m^2)	22.7 ± 3.5	23.3 ± 3.2	0.20
VFA (cm^2)	75.7 ± 50.8	93.5 ± 53.5	0.08
FBS (mg/dL)	112.6 ± 26.9	108.7 ± 31.1	0.44
HbA1c (%)	5.7 ± 1.2	5.6 ± 1.0	0.43
Leptin (ng/mL)	5.6 ± 4.3	5.4 ± 4.2	0.70

Data are shown as mean ± standard deviation. Statistical analysis was performed using the Mann–Whitney *U*-test. *$P < 0.05$. **$P < 0.01$. BMI, body mass index; VFA, visceral fat area; FBS, fasting blood sugar.

Results

Serum leptin levels and colorectal tumors. The clinical characteristics of the colorectal adenoma patients and control subjects without colorectal polyps are shown in Table 1. No significant difference in the mean serum leptin level was observed between the two groups. There were also no significant differences in age, BMI or other obesity-related factors between the two groups. A good correlation was observed between the BMI and serum leptin levels ($R = 0.533$, $P < 0.01$) (Fig. 1a), as previously reported.[7–9] We also investigated the differences in the serum leptin levels depending on the tumor size and pathological grade; however, no correlations were observed (Fig. 1b,c).

Leptin receptor, ObR, expression in the colorectal adenoma and normal colorectal tissues. To examine the ObR expression in colorectal adenoma and normal colorectal tissues, immunohistochemical staining and gene expression analyses were performed. ObR was clearly expressed in the cytoplasm of the colorectal adenoma gland cells, but only slightly in the normal colorectal gland cells in the vicinity of the adenomas (Fig. 2a–f). The frequency of detection of ObR in the colorectal adenomas was 67.2% (41/61). For the present study, no isoform-specific antibodies for ObRL and ObRS were available. Therefore, we

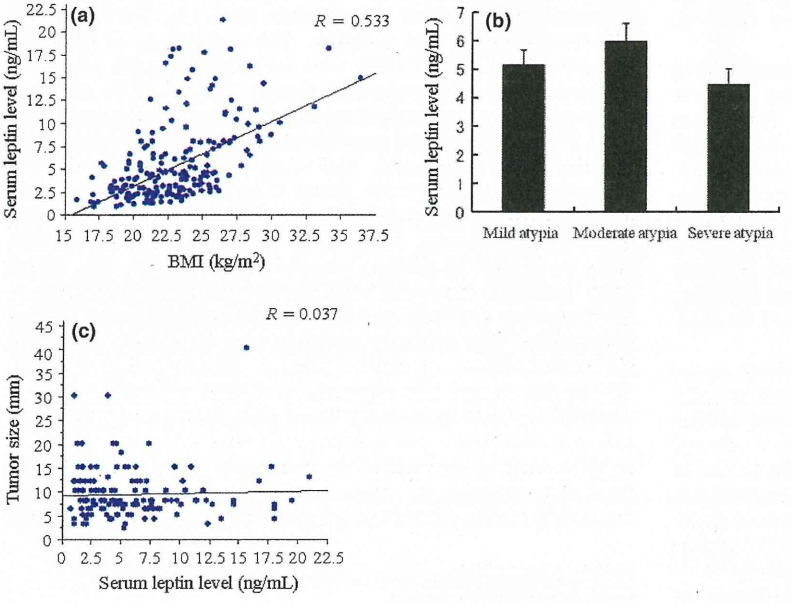

Fig. 1. Correlation between serum leptin levels and body mass index (BMI), tumor pathology and size. (a) Correlation between serum leptin levels and BMI. Each point represents each individual patient ($P < 0.01$, $R = 0.533$). (b) Correlation between serum leptin levels and pathological grade (mild, moderate and severe atypia). Each column represents the mean ± SEM from 23 to 65 patients. (c) Correlation between serum leptin levels and tumor size. Each point represents each individual patient ($P = 0.664$, $R = 0.037$).

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