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Effect of hepatocyte growth factor on endogenous hepatocarcinogenesis in rats fed a choline-deficient L-amino acid-defined diet

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Received December 13, 2005; Accepted February 15, 2006

Abstract. Hepatocyte growth factor (HGF) is a promising agent for the treatment of intractable liver disease, due to its mitogenic, anti-apoptotic, and anti-fibrotic effects. We investigated the effect of recombinant human HGF (rh-HGF) on the development of both hepatocellular carcinoma (HCC) and preneoplastic nodules in rats fed a choline-deficient L-amino acid-defined (CDAA) diet, an animal model of hepatocarcinogenesis resembling human development of HCC with cirrhosis. From weeks 13 to 48 of the CDAA diet, rh-HGF (0.1 or 0.5 mg/kg/day) was administered intravenously to rats in four-week cycles, with treatment for five consecutive days of each week for two weeks, followed by a two-week washout period. Treatment with rh-HGF significantly inhibited the development of preneoplastic nodules in a dose-dependent manner at 24 weeks. Although the numbers and areas of the preneoplastic nodules in rats treated with rh-HGF were equivalent to those in mock-treated rats by 60 weeks, the incidence of HCC was reduced by HGF treatment. Although one rat treated with low-dose rh-HGF exhibited a massive HCC, which occupied almost the whole liver, and lung metastases, HGF treatment did not increase the overall frequency of HCC. Administration of high-dose rh-HGF, however, induced an increase in the urinary excretion of albumin, leading to decreased serum albumin at 60 weeks. These results indicate that long-term administration of rh-HGF does not accelerate hepatocarcinogenesis in rats fed a CDAA diet. However, these findings do not completely exclude the potential of HGF-induced hepatocarcinogenesis; this issue must be resolved before rh-HGF can be used for patients with intractable liver diseases, especially those with cirrhosis.

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Key words: hepatocyte growth factor, hepatocarcinogenesis, choline-deficient, L-amino acid

Introduction

Hepatocyte growth factor (HGF), originally isolated from the plasma of patients with fulminant hepatic failure, was identified as a potent mitogen for hepatocytes (1,2). HGF is a multifunctional growth factor that acts as a mitogen, motogen, and morphogen for a wide variety of cells, including epithelial and endothelial cells (3-6). In addition to promoting hepatocyte proliferation (7-9), this factor acts in concert with transforming growth factor (TGF)-α and heparin-binding epidermal growth factor during liver regeneration (10,11). HGF also ameliorates hepatic injury by stimulating anti-apoptotic effects in animal models of fulminant hepatic failure (12-18) and attenuating hepatic fibrosis in animals with liver cirrhosis (19-23). Consequently, HGF may induce liver regeneration, inhibit disease progression, and ameliorate hepatic fibrosis in patients suffering from intractable liver disease.

We have established an enzyme-linked immunosorbent assay to measure serum levels of human HGF. Using this assay, we identified increased serum HGF levels in patients with a variety of liver diseases (24). Serum HGF levels are a valuable prognostic tool in fulminant hepatic failure (25). We attempted to develop an innovative therapy using recombinant human HGF (rh-HGF) for the treatment of fatal liver diseases, including fulminant hepatic failure, small-for-size liver grafts in living donors, and liver cirrhosis. We recently demonstrated that bolus intravenous injection of rh-HGF led to increased serum levels of human HGF, which primarily distributed to the

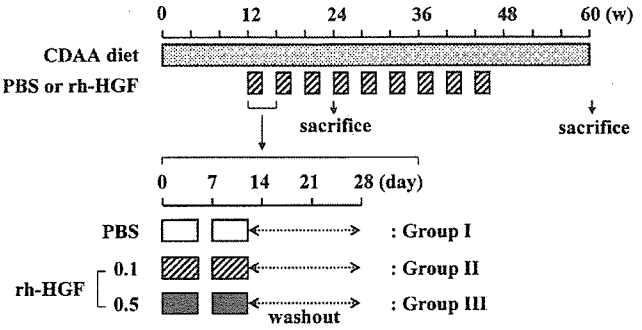

Figure 1. Experimental protocol. After a one-week acclimation period, rats were fed a CDAA diet for 60 weeks. After 12 weeks of diet administration, rh-HGF (0.1 or 0.5 mg/kg/day) or PBS was injected intravenously into rats for five consecutive days of the week for two weeks, followed by a two-week washout period. This four-week treatment was repeated from weeks 13 to 48 of CDAA diet administration. Rats were sacrificed at 24 and 60 weeks.

liver (26). Despite its short half-life, a single intravenous injection of rh-HGF induced the tyrosine phosphorylation of c-Met, the specific receptor for HGF, in liver tissues. It is necessary, however, to resolve whether repeated intravenous injection of rh-HGF accelerates hepatocarcinogenesis before clinical application of rh-HGF can proceed to the treatment of intractable liver diseases, especially cirrhosis, in which hepatocellular carcinoma (HCC) develops at a high incidence.

In rats fed a choline-deficient L-amino acid-defined (CDAA) diet, HCC develops without any exposure to exogenous carcinogens (27). This animal model of hepatocarcinogenesis is an appropriate experimental system, as HCC occurs in conjunction with fatty liver, hepatocyte death, and subsequent regeneration, fibrosis, and eventual cirrhosis (28), a similar progression to the histopathological sequence of human HCC development with cirrhosis.

In this study, we administered rh-HGF intravenously to rats fed a CDAA diet for an extended period (36 weeks). We then investigated the effect of HGF treatment on the development of preneoplastic nodules and HCC, which arises from neoplastic nodules, induced by long-term (60 weeks) administration of a CDAA diet.

Materials and methods

Animals. Six-week-old male Fischer 344 rats were obtained from Kyushu Experimental Animal Supply (Kumamoto, Japan). Animals were maintained at a constant room temperature (25°C) and provided free access to water and the food indicated throughout the study. The protocols for these animal studies were approved by the ethics committee of the University of Miyazaki (Miyazaki, Japan).

Experimental protocol. After a one-week acclimation period on a standard diet, rats were switched to a CDAA diet (Dyets Inc., Bethlehem, PA). After a 12-week administration of the CDAA diet, rh-HGF (0.1 or 0.5 mg/kg/day) (Mitsubishi Pharma Co., Tokyo, Japan) or phosphate-buffered saline (PBS) was injected intravenously into rats on five consecutive days of a week for two weeks, followed by a two-week washout from rh-HGF or PBS treatment (Fig. 1). This four-week treatment was repeated from weeks 13 to 48 of the CDAA diet administration. The rats were sacrificed at weeks 24 and 60. Blood was obtained from the bifurcation of the abdominal aorta, from which we determined platelet counts, and the serum levels of alanine aminotransferase (ALT), albumin, total cholesterol (T-Chol), hyaluronic acid, and creatinine. We also measured the urinary excretion of albumin. The liver, spleen, and bilateral kidneys were immediately excised after sacrifice; the wet weight of these organs was then determined. Samples were subjected to histological analysis or frozen in liquid nitrogen and stored at -80°C until analysis. After 60 weeks of CDAA diet administration, rats were maintained in metabolic cages, allowing quantitative urine collection for three consecutive days that was used for the measurement of albumin excretion.

Table I. Effect of rh-HGF administration on the weights of the total body, liver, and kidneys in rats fed a CDAA diet for 60 weeks.

Group	n	Body (g)	Liver (g/100 g body wt.)	Kidneys (g/100 g body wt.)
I	14	368±62	4.43±0.45	0.69±0.07
II	16	369±46	4.57±0.72	0.72±0.09
III	13	300±51[a]	5.56±1.82[b]	0.93±0.15[c]

The data are mean ± SD. [a]p=0.0053 or 0.0038 vs. I or II, respectively; [b]p=0.0403 vs. I; [c]p<0.0001 vs. I and II.

Histopathological and immunohistochemical analysis. To evaluate the development of hepatocellular carcinoma (HCC), 5-mm thick slices of whole liver were fixed in 10% formalin and embedded in paraffin. A 2-μm section, prepared from each fixed liver slice, was then stained with hematoxylin and eosin (H&E). Histological examinations were performed independently by two investigators blinded to the protocol. HCC was diagnosed according to well-established criteria (29).

We examined the development of precancerous lesions, which are positive for the placental form of rat liver glutathione S-transferase (GST-P), in three 5-mm thick slices obtained from the three major liver lobes (left lateral and the left and right median lobes). A 4-μm section prepared from each fixed liver slice was subjected to immunohistochemical analysis as described (28). Briefly, after boiling in distilled water for 10 min, slides were incubated with a rabbit polyclonal antibody against GST-P (Medical and Biological Laboratories, Nagoya, Japan). After application of goat anti-rabbit IgG (Nichirei Co., Tokyo, Japan), slides were treated with avidin-biotin-peroxidase complex and chromatin 3',3'-diaminobenzidine. The number of GST-P positive nodules was counted; the area of each nodule was measured using Image-Pro Plus software (version 4.5.1.28; Media Cybernetics Inc, MD, USA).

RNA isolation and quantitative reverse transcription (RT)-PCR. We utilized quantitative PCR to examine the expression of albumin mRNA. Total RNA was extracted from rat liver tissues using Isogen reagent (Nippon Gene Co., Toyama,

Table II. Effect of rh-HGF on biochemical markers in rats fed a CDAA diet for 60 weeks.

Group	n	ALT (IU/L)	s-Alb (g/dl)	T-Chol (mg/dl)	Plt (10^4/mm^3)	HA (ng/ml)	Cre (mg/dl)	u-Alb (mg/day)
I	14	99±31	4.4±0.3	68±15	58.1±10.1	47±7	0.28±0.08	33.3±11.6
II	15	126±83	4.0±0.4	87±35	59.0±10.0	56±18	0.29±0.10	40.9±20.5
III	13	65±22[a]	3.2±0.6[b]	135±30[c]	70.0±14.8[d]	66±37	0.21±0.15	127.3±18.3[b]

The data are mean ± SD. ALT, alanine aminotransferase; s-Alb, serum albumin; T-Chol, total cholesterol; Plt, platelet count; Cre, serum creatinine; u-Alb, urinary excretion of albumin. [a]p=0.0188 vs. II; [b]p<0.0001 vs. I and II; [c]p<0.0001 or =0.0003 vs. I or II, respectively; [d]p=0.0425 vs. I.

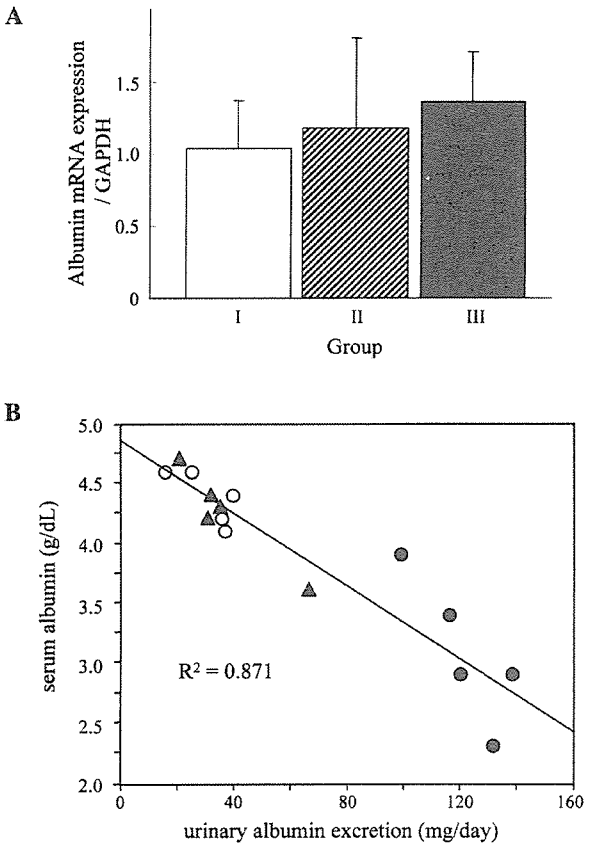

Figure 2. Hepatic expression of albumin mRNA in rats fed a CDAA diet, and the relationship between serum levels and urinary excretion of albumin. (A) Expression of albumin mRNA in liver tissues was examined by real-time RT-PCR. In comparison to mock-treated rats [group I (n=5); open column], albumin expression was unaffected by HGF administration [group II (n=5) and III (n=5); hatched and closed columns, respectively]. (B) Serum levels of albumin inversely correlated with urinary excretion of albumin. Group I, open circles; group II, triangles; group III, closed circles.

Japan). Total RNA (0.5 μg) was reverse transcribed using random hexamer priming in the presence of MMLV reverse transcriptase. PCR reactions combined TaqMan Universal PCR master mix, containing PCR primers and fluorogenic probes specific for rat albumin (Applied Biosystem, Foster City, CA), and 2.5 μl cDNA in a total volume of 25 μl. PCR amplification was performed in triplicate using the following temperature and cycling profile: after an initial incubation at 50°C for 2 min and then at 95°C for 10 min, we performed 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Albumin transcripts, quantitated using an ABI PRISM 7700 Sequence detection system (Applied Biosystem), were normalized to the levels of amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Statistical analysis. Statistical analysis was performed using Statview J-4.5 software (Abacus Concepts, Inc., Berkeley, CA). Data are expressed as means ± SD. Normally distributed variables in different groups were compared by analysis of variance, while comparisons were performed using Fisher's PLSD or the Scheffè F test. The inter-group difference was considered to be statistically significant when the p-value was <0.05.

Results

Effect of rh-HGF on the weights of the body, liver, and kidneys in rats fed a CDAA diet. The weights of the total body, the liver, and the bilateral kidneys were measured after CDAA administration for 60 weeks (Table I). Repeated injections of rh-HGF at a low dose (0.1 mg/kg/day) (group II) did not affect these values in comparison to the mock-treated rats (group I). Rats treated with a high dose (0.5 mg/kg/day) of rh-HGF (group III); however, exhibited a significant decrease in body weight and a significant increase in the weights of the liver and kidneys.

Effect of rh-HGF on biochemical markers in rats given a CDAA diet. We also examined biochemical markers, platelet counts, and urinary excretion of albumin in rats fed the CDAA diet for 60 weeks (Table II). When compared to the mock-treated group (group I), administration of rh-HGF at a low dose (group II) did not significantly impact the expression of biochemical marker, platelet counts, or urinary excretion of albumin. In group III, however, administration of rh-HGF at a high dose decreased serum ALT levels and increased T-Chol and platelet counts. The serum levels of hyaluronic acid and creatinine were not affected. Decreases in serum albumin and increases in urinary excretion of albumin were also induced by this treatment. This result prompted us to examine hepatic expression of albumin and the relationship between serum levels and urinary excretion of albumin (Fig. 2). Although treatment with rh-HGF did not affect albumin expression in the liver (Fig. 2A), we observed an inverse correlation between

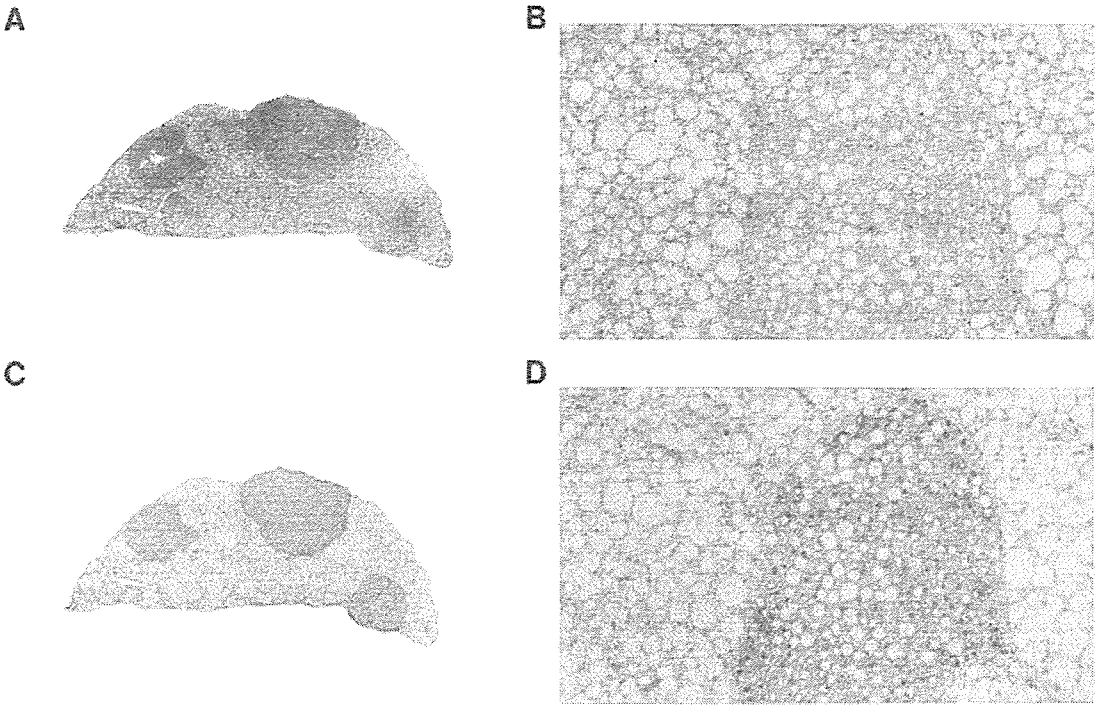

Figure 3. Representative photographs of liver derived from a rat fed a CDAA diet for 24 weeks. A section of liver tissue was stained with both H&E (A and B) and an anti-GST-P antibody (C and D). The development of liver cirrhosis and GST-P-positive preneoplastic nodules were observed in rats fed a CDAA diet for 24 weeks (group I) [original magnifications x100 (B and D)].

serum levels and urinary excretion of albumin (Fig. 2B). These results indicate that the observed decrease in serum albumin results primarily from the urinary loss of albumin, which is induced by repeated administration with high-dose rh-HGF.

Administration of rh-HGF suppressed the early development of preneoplastic lesions in rats fed a CDAA diet. In rats given a CDAA diet, collagen fibers began to extend at four weeks, which was shortly followed by the development of cirrhosis.

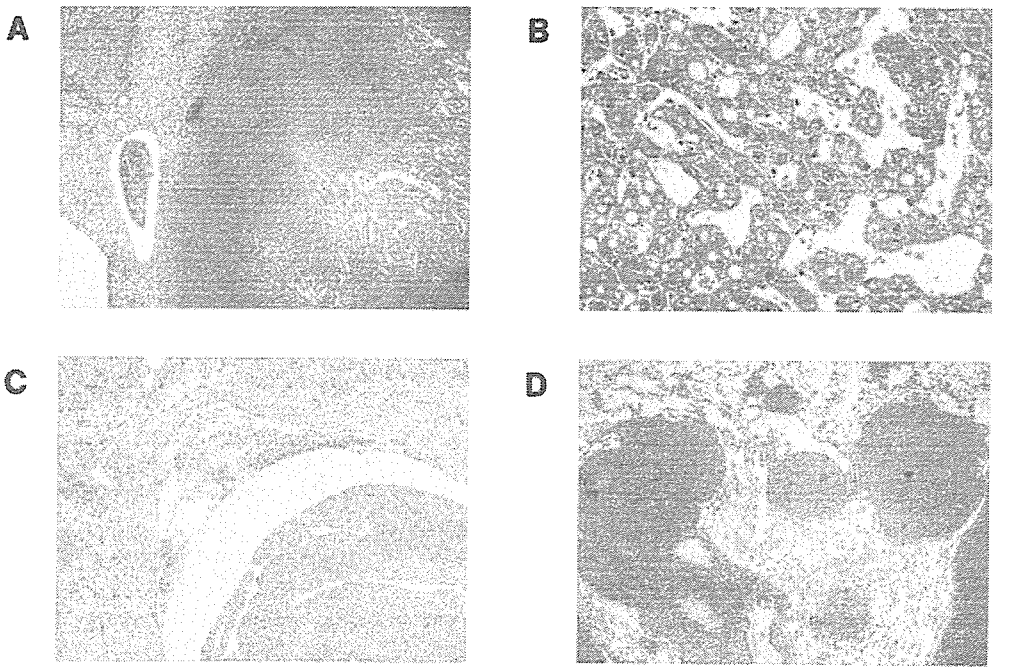

Figure 4. Representative microphotographs of the HCCs and metastatic lung tumors developing in rats treated with rh-HGF. (A and B) HCC lesions developed in rats fed a CDAA diet, who were treated with high-dose rh-HGF (group III) [magnifications x40 (A) and x400 (B)]. Cellular atypia, irregular shapes, and trabecular patterns were observed. Necrosis was also present within the HCC lesion. One of the seven animals treated with low-dose rh-HGF exhibited a large HCC lesion, which occupied the majority of the liver and invaded into the large hepatic vein (C), as well as lung metastases (D) [magnifications x40 (C and D)].

Table III. Effect of rh-HGF administration on the development of GST-P-positive preneoplastic nodules in rats fed a CDAA diet.

	24 weeks				60 weeks			
Group	n	Number/body	Area (%)	p-value (vs. I)	n	Number/body	Area (%)	p-value (vs. I)
I	4	17.5±4.7	20.2±8.0	-	14	44.2±8.7	20.3±9.3	-
II	4	15.3±3.3	7.4±4.2	0.033	16	40.6±16.8	21.7±12.0	0.917
III	4	18.0±2.9	8.0±3.7	0.040	13	34.2±13.5	16.6±5.6	0.606

The data are mean ± SD.

Table IV. Effect of rh-HGF on HCC development in rats fed a CDAA diet for 60 weeks.

		Incidence			
Group	n	n (%)	p-value (vs. I)	Number/ body	HCC area (%)
I	14	8 (57.1)		1.8±0.9	1.5±1.8
II	16	7 (43.8)	0.464	2.1±1.9	7.9±14.4
III	13	3 (23.1)	0.071	1.3±0.6	0.6±0.4

The data are mean ± SD.

Preneoplastic nodules positive for GST-P were observed in a small number of rats fed the CDAA diet for four weeks (28). In this study, all rats exhibited liver cirrhosis and GST-P-positive nodules by 24 weeks (Fig. 3). To determine the effects of rh-HGF administration on the development of GST-P-positive nodules, we examined the numbers and areas of preneoplastic nodules positive for GST-P in rats given a CDAA diet at 24 and 60 weeks (Table III). Although treatment with rh-HGF did not affect the number of GST-P positive nodules at 24 weeks, the average areas of these nodules were reduced following both low- and high-dose rh-HGF administration. At 60 weeks of CDAA diet administration, treatment with low-dose rh-HGF (group II) did not affect the number or area of GST-P positive nodules from the values observed in group I. High-dose rh-HGF administration, however, appeared to inhibit the development of GST-P-positive nodules, although there was no statistical significance of this inhibition in comparison to either group I or II.

Treatment with rh-HGF does not accelerate development of HCCs in rats given a CDAA diet for 60 weeks. Multiple GST-P-positive nodules develop into HCCs during a long-term administration of a CDAA diet. These developing HCCs, which exhibit cellular atypia, irregular shapes, and trabecular patterns (Fig. 4A and B), were observed in a proportion of the rats at 60 weeks. To clarify the effect of rh-HGF administration on HCC development, we examined the incidence, number, and area of HCC lesions in rats fed a CDAA diet for 60 weeks (Table IV). Although these values were not statistically significant, the incidence of HCCs was reduced in a dose-dependent manner following HGF treatment. The number of HCC lesions per animal was not affected by rh-HGF administration, regardless of the dose. In comparison to rats in group I with HCCs, treatment with high-dose rh-HGF did not affect the average area of HCC lesions in tumor-bearing rats. In group II, one of the seven rats exhibited massive HCCs, which occupied the majority of the liver and invaded into the large hepatic vein, in addition to a number of lung metastases (Fig. 4C and D). This animal increased the average HCC area of this group significantly (Table IV).

Discussion

Increased rates of benign and malignant tumor formation occur in the livers and mammary glands of HGF transgenic mouse strains (30-33). In addition, both diethylnitrosamine-induced hepatocarcinogenesis and ultraviolet radiation-induced skin carcinogenesis are accelerated in these mice (33-35), suggesting that prolonged and continuous exposure to HGF may accelerate neoplastic development in multiple organs. Transgenic mice specifically overexpressing HGF in the liver, however, do not develop HCC; the development of hepatic neoplasms induced by either TGF-α or c-myc overexpression was inhibited in these mice (36,37). Several investigators have examined the effect of a recombinant form of HGF lacking five amino acids (dHGF) (38) on the development of preneoplastic nodules or HCC in rat models of carcinogen-induced hepatocarcinogenesis. Although administration of recombinant dHGF stimulated DNA synthesis in preneoplastic nodules, this treatment inhibited cell proliferation of HCCs in a rat model of hepatocarcinogenesis induced by either diethylnitrosamine (DEN) or 3'-methyl-4-dimethylaminoazobenzene (39,40). Yaono *et al* reported that recombinant dHGF enhanced the development of preneoplastic hepatic foci in rats treated with the combination of DEN and N-ethyl-N-hydroxyethylnitrosamine (41). In this study, we administered rh-HGF to a rat model of hepatocarcinogenesis induced by the CDAA diet. Treatment with rh-HGF inhibited the development of preneoplastic nodules at 24 weeks of diet administration. As not all neoplastic nodules develop into HCC, we evaluated the effect of HGF administration on the development of HCC at 60 weeks. Despite the fact that these values were not statistically significant, HGF treatment reduced the overall incidence of HCC development. Although HGF treatment did not affect the development of preneoplastic nodules at 60 weeks, the inhibition of preneoplastic nodule development at 24 weeks

may contribute to the reduced incidence of HCC in rh-HGF rats at CDAA-60 weeks. HGF is known to function as both a mitogenic and anti-apoptotic agent, but also appears to induce apoptosis in a subset of malignant cells, such as sarcomas (42,43). Oxidative stress is likely involved in mechanism by which HGF suppresses the growth of tumor cells (44). Therefore, HGF may act as an apoptotic agent *in vivo* for HCC cells developing within the livers of rats fed a CDAA diet. The precise mechanism governing these reciprocal functions, mitogenesis and the induction of apoptosis in non-malignant and malignant cells, respectively, remains poorly understood.

One of seven rats bearing HCCs in group II exhibited a number of massive HCCs, which occupied the majority of the liver, in the presence of lung metastases. The incidence of HCC tended to be reduced following HGF treatment in comparison to mock-treated rats (group I). In addition, the number and areas of the observed HCC lesions were not increased by treatment with high-dose HGF. Therefore, administration of low-dose rh-HGF was not associated with the massive enlargement of HCC lesion, which was observed in only a single rat of group II. As HGF is known to act as a scattering factor, intravenous administration of rh-HGF may have influenced the development of metastatic lung neoplasia.

HGF reduces hepatic fibrosis in animal models of liver cirrhosis (19-23). This study is the first in which rh-HGF was administered to rats fed a CDAA diet. Administration of rh-HGF did not facilitate the hepatic fibrosis induced by the CDAA diet (data not shown). Although both liver weight and platelet counts were increased and serum ALT levels were reduced following treatment with high-dose rh-HGF (group III; 0.5 mg/kg/day), rh-HGF treatment may not be sufficient to attenuate the hepatic fibrosis induced by a CDAA diet. In contrast, long-term administration of high-dose rh-HGF (group III) induced a marked increase in the urinary excretion of albumin. Although serum creatinine levels were not affected by rh-HGF treatment, this albuminuria was irreversible, continuing for 12 weeks after the discontinuation of rh-HGF administration. Preliminary experiments revealed that repeated injections of rh-HGF for two weeks increased the urinary excretion of albumin; this albuminuria disappeared almost completely within one to two weeks after washout of rh-HGF (45). The histopathological findings seen in the kidneys after a two-week administration of rh-HGF were indicative of reversible changes. We therefore administered rh-HGF for five consecutive days of each week for two weeks followed by a two-week washout period to prevent rh-HGF-mediated nephrotoxicity (Fig. 1). In our studies, rats treated with low-dose rh-HGF (group II; 0.1 mg/kg/day) exhibited albuminuria equivalent to mock-treated rats (group I) by 60 weeks (12 weeks after washout of rh-HGF treatment). Conversely, as the serum levels of rh-HGF increase following intravenous administration in a bolus and the half-life of this molecule is prolonged in cirrhotic rats (26,45), rh-HGF nephrotoxicity may be augmented in rats fed a CDAA diet. Further experimentation is necessary to clarify the safe doses of rh-HGF that can be administered long-term or to patients with severe liver disease.

We are in the process of preparing a clinical study of rh-HGF administration to patients in fulminant hepatic failure, which is an intractable and fatal disease. The preliminary results of this clinical study indicate that short-term administration of rh-HGF is sufficient to rescue the patients, indicating a minimal possibility of HGF-induced hepatocarcinogenesis. HCCs, however, frequently develops in cirrhotic livers; long-term administration of rh-HGF is required to attenuate hepatic fibrosis in these patients. Therefore, it is important to resolve if long-term administration of rh-HGF accelerates the development of malignant tumors before we begin clinical trials for patients with liver cirrhosis. In this study, we administered rh-HGF for 36 weeks to rats fed a CDAA diet, an animal model of hepatocarcinogenesis resembling the development of human HCC. We then evaluated the effects of HGF treatment on the development of preneoplastic nodules and HCC lesions over a long experimental period. Although HGF treatment did not accelerate HCC development in this rat model of endogenous hepatocarcinogenesis, further *in vivo* investigations are required to clarify the risk of carcinogenesis induced by rh-HGF in both the liver and non-hepatic organs.

Acknowledgements

We thank Ms. Yuko Nakamura, Ms. Sayoko Ohara, and Ms. Kana Ohara for their technical assistance. This study was supported in part by Health and Labor Science Research Grants from the Ministry of Health, Labor, and Welfare of Japan for the Research of Intractable Diseases.

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Scandinavian Journal of Gastroenterology, 2006; 41: 312–317

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ORIGINAL ARTICLE

Protective effects of heat shock protein 70 induced by geranylgeranylacetone on oxidative injury in rat intestinal epithelial cells

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Abstract

Objective. Geranylgeranylacetone (GGA), an anti-ulcer agent, has recently been demonstrated to protect a variety of cells and tissues via induction of heat shock protein (HSP)70 against numerous stresses. We investigated whether GGA induces HSP70 and protects against an oxidative stressor, monocrolamine (NH_2Cl), in a rat intestinal epithelial cell line (IEC-18). ***Material and methods***. IEC-18 cells pretreated with GGA (0.1–10 μM) were subjected to injury induced by NH_2Cl. Cell viability was assessed, and endogenous HSP70 levels were determined by enzyme-linked immunosorbent assay in IEC-18 cells. ***Results***. Treatment with GGA (0.1–10 μM) was found rapidly to elevate HSP70 levels and to protect against NH_2Cl-induced injury in IEC-18 cells. Furthermore, quercetin, an inhibitor of HSP70 synthesis, diminished the protective effects of GGA in IEC-18 cells upon NH_2Cl-caused injury. ***Conclusions***. The results of this study suggest that GGA plays an important role in defense mechanisms against oxidative injury in the intestine, primarily via induction of HSP70.

Key Words: *Geranylgeranylacetone, heat shock protein, intestinal epithelial cells, oxidative injury*

Introduction

Intestinal epithelial cells (IECs) play an important role in the defense system against toxins and antigens produced by bacteria and food. Disorders of this defense system lead to intestinal injury and inflammation such as that occurring in inflammatory bowel diseases [1]. Oxidative injury is one of the major injurious factors in the development of intestinal inflammation [2]. In particular, monochrolamine (NH_2Cl) is known to be an oxidative reagent and plays an important physiological role in the development of intestinal inflammation and tissue damage [2]. Based on these findings, NH_2Cl was selected as a reproducible model of oxidant-induced injury that mimics the effects of the toxic and oxidizing agents produced by activated inflammatory cells [2,3].

The defense system against injury and stress is important in the survival of intestinal cells. Once cells are exposed to various stresses, cells preferentially synthesize heat shock proteins (HSPs) required to survive under stress conditions such as heat, anoxia, ultraviolet light, and oxidative agents [4,5]. HSPs are ubiquitous proteins synthesized by a variety of stresses, including heat shock, and play a critical role in the maintenance of cell integrity under pathophysiological conditions [5]. In cytoprotective HSPs, HSP70 belongs to the HSP70 family, and is known to play an important role in cell protection in many tissues [5]. In the intestine, thermal or chemical-induced HSP70 protects intestinal epithelial cells from oxidative injury *in vitro* and *in vivo* [6,7].

Geranylgeranylacetone (GGA) is an anti-ulcer drug, developed in Japan [8], that protects gastric epithelial cells from various stresses without affecting gastric acid secretion [9–12]. GGA has been con-

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(Received 6 May 2005; accepted 18 August 2005)

ISSN 0036-5521 print/ISSN 1502-7708 online
DOI: 10.1080/00365520500319427

sidered to play a central role in mucosal cytoprotection of the stomach, independently of endogenous prostaglandins [13]. Interestingly, a recent study by Hirakawa et al. has shown that GGA directly and selectively induces HSP70 in gastric mucosa cells and protects the cells against several stresses such as hydrochloride and indomethacin [14]. In other experimental models, HSP70 induced by GGA has been found to protect cells and tissues from injury or ischemia [15–17]. In intestinal cells, Tsuruma et al. have demonstrated that GGA induced HSP70 in the mucosa of rat intestine [18]. There has been no report, however, regarding the protective effect of GGA on intestinal epithelial cells.

In the present study, to elucidate the effects of GGA on intestine, we investigated whether GGA could induce the expression of HSP70 and protect against oxidative stress by NH_2Cl in rat intestinal epithelial cells, IEC-18 cells. We demonstrated for the first time that GGA markedly elevates the level of HSP70 and protects IEC-18 cells against oxidative injury caused by NH_2Cl. Our results indicate that treatment with GGA may be useful for intestinal injury such as inflammation.

Material and methods

Chemicals

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin solution were purchased from the Sigma Chemical Co. (St Louis, Mo., USA); the enzyme-linked immunosorbent assay (ELISA) kit for HSP70 was from Stressgen (Victoria, BC, Canada); the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was from Promega (Madison, Wisc., USA). GGA and α-tocopherol were kindly provided by the Eisai Co. (Tokyo, Japan).

Cell culture

IEC-18 cells were obtained from the American Type Culture Collection (ATCC; Rockville, Md., USA) and cultured in DMEM containing 5% heat-inactivated FBS, 0.1 ml insulin, 50 μg/ml streptomycin, and 50 U/ml penicillin. IEC-18 cells from 15–20 passages were used in the experiments.

Treatment of cells with GGA or monochrolamine

GGA was dissolved in absolute ethanol containing 0.25% α-tocopherol and diluted with phosphate buffered saline (PBS). Vehicle was PBS containing 0.25% α-tocopherol without GGA. For treatment with GGA, cells were incubated with 0.1, 1, and 10 μM GGA for 60–240 min. After incubation with GGA, the cells were washed with PBS and cultured with DMEM, and then then incubated with NH_2Cl at concentrations of 0.1, 0.3, and 1 mM for 60 min. NH_2Cl was prepared just before each use by a previously described method [19].

Examination of cell viability

To examine the protective effects of GGA on IEC-18 cells against oxidant-induced injury, cell viability was assessed by XTT assay. XTT assay is a non-radioactive assay that correlates well with the percentage of ^{51}Cr release from the rat intestinal epithelial cells for evaluation of cell viability, as described previously [20]. In the present study, XTT assay was performed with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) in accordance with the manufacturer's instructions. Briefly, cells were grown to an approximate cell concentration of 10^4 cells/well in 96-well plates. Cells in each well were incubated at 37°C with 150 μl DMEM and 50 μl CellTiter 96 Aqueous One Solution Cell Proliferation reagent. Cell viability was determined by the generation of a formazan dye from the substrate. Optical density (OD) was measured by spectrometer 0 and 90 min after the addition of CellTiter 96 Aqueous One Solution Cell Proliferation reagent. The difference in OD between 0 and 90 min after incubation was calculated to evaluate cell viability. The assay was run on cells without GGA and NH_2Cl exposures as a negative control. All experiments were repeated more than three times to confirm reproducibility.

ELISA for HSP70

For assessment of HSP70 induction in cells, the levels of endogenous HSP70 in cells were measured by ELISA for HSP70. Cells were washed with PBS and homogenized with a sonicator at 4°C. The homogenates were centrifuged at 27,000*g* for 10 min, and the supernatants were collected. The supernatants were dissolved in PBS containing a cocktail of protease inhibitors (1 μl to 20 mg of tissue according to the manufacturer's protocol). Protein concentrations were measured with a BCA microprotein kit, in accordance with the manufacturer's protocol. After the protein concentrations of the samples were equally adjusted, HSP70 was measured using an ELISA kit specific for rat HSP70 according to the manufacturer's protocol. Briefly, 100 μl of the sample was incubated in each well of a 96-well plate for 2 h at room temperature. After the plate was washed six times with washing buffer, 100 μl biotin-conjugated anti-HSP70 antibody solution was added to each well. Following incubation

for 1 h at room temperature, the plate was again washed six times with the washing buffer. The avidin-conjugated goat anti-rabbit IgG antibody was then added to each well, and the microtiter plate was incubated for 1 h at room temperature. After washing six times with the washing buffer, the substrate solution (100 μl) was added to each well. The substrate solution (10 μl) contained 8 μg o-phenylenediamine and 4 μl of 30% H_2O_2 in citrate phosphate buffer (pH 5.0). After incubation for 10 min at room temperature, the reaction was terminated with 25 μl of 4N sulfuric acid. The absorbance was measured at 450 nm using an ELISA plate reader (Model 3550; Bio-Rad, Hercules, Calif., USA). Recombinant HSP70 protein was used to obtain the standard curve in this experiment.

Treatment with quercetin, an HSP70 synthetic inhibitor

Quercetin has been shown to inhibit the production of HSP70 [6,21]. To investigate the effects of quercetin, cells were pretreated with quercetin at a final concentration of 200 μM for 2 h before GGA treatment. Cell viability was determined by XTT assay, and HSP70 levels were measured by ELISA in cells pretreated with quercetin.

Statistical analysis

All results are expressed as the mean±standard deviation (SD) and were statistically analyzed using Student's *t*-test with Bonferroni's correction (StatView, SAS Institute, Cary, N.C., USA). A *p*-value of <0.05 was considered to be statistically significant.

Results

Effect of GGA on cell protection against oxidative stress in IEC-18 cells

Morphological alteration and growth inhibition were not observed in the IEC-18 cells after exposure to GGA or vehicle (data not shown). To evaluate the effects of GGA against oxidative stress, cell viability in IEC-18 cells treated with NH_2Cl was analyzed by XTT assay. The difference in OD, as a parameter of cell viability, was significantly decreased in the cells treated with NH_2Cl in a dose-dependent manner (0 mM, 0.91±0.06, 0.1 mM, 0.67±0.03, 0.3 mM, 0.47±0.07, and 1 mM, 0.40±0.15). In the present study, we used NH_2Cl at a concentration of 0.3 mM for oxidative stress. In the dose–response analysis, we found that 0.1, 1, and 10 μM GGA (0.59±0.03, 0.65±0.07, and 0.67±0.04, respectively, $p<0.05$ (0.1 μM) and $p<0.01$ (1 and 10 μM) versus vehicle- and NH_2Cl-treated cells) significantly increased cell viability in the cells 90 min after GGA treatment compared with vehicle-treated cells (0.42±0.03) (Figure 1a). In the time-course analysis, cell viability in the IEC-18 cells was remarkably improved 60, 90, 120, and 240 min after treatment with 1 μM GGA (0.66±0.06, 0.69±0.06, 0.72±0.12, and 0.70±0.08, respectively, $p<0.01$ versus vehicle- and NH_2Cl-treated cells) (Figure 1b).

Effect of GGA on induction of HSP70 in IEC-18 cells

The levels of endogenous HSP70 in the cells exposed to vehicle were essentially unchanged compared with the non-treated cells (17.4±5.0 and 18.4±2.9 ng/mg protein, respectively). GGA at concentrations of 0.1, 1, and10 μM significantly increased the HSP70 levels in IEC-18 cells compared with vehicle-treated cells (42.1±8.2,

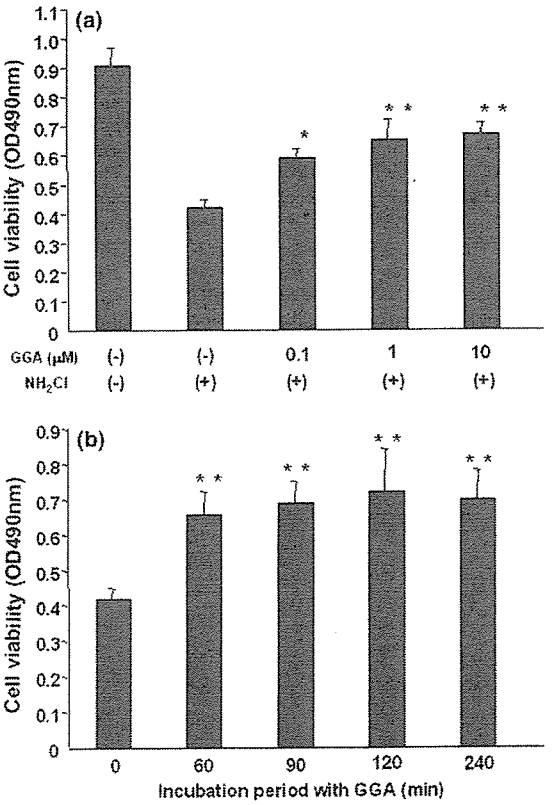

Figure 1. Effects of geranylgeranylacetone (GGA) on the protection of intestinal epithelial cell (IEC)-18 cells from monochrolamine. (a) IEC-18 cells were incubated with the indicated concentrations of GGA for 90 min in the presence of monochrolamine (0.3 mM). (b) After cells were incubated with GGA (1 μM) for 60, 90, 120, and 240 min, monochrolamine (0.3 mM) was directly added to the culture medium and incubated for 1 h. The cell viability was then determined by XTT assay. Values are means±standard deviation (SD). N=5; *$p<0.05$; **$p<0.01$ versus vehicle and NH_2Cl-treated cells.

56.2±7.1, and 48.4±9.7 ng/mg protein, $p < 0.01$ vs. vehicle-treated cells) (Figure 2a) at 60 min after GGA treatment. There was no significant difference in HSP70 levels among 0.1, 1, and 10 μM GGA-treated cells.

In the time-course study, the HSP70 levels rapidly increased 60 min after 1 μM GGA treatment in IEC-18 cells (55.2±14.2 ng/mg protein). The increase in HSP70 levels in the cells peaked at 90 min (60.6±19.8 ng/mg protein) and remained at more than 2-fold the initial levels at 240 min after GGA treatment compared with vehicle-treated cells. (56.2±12.5 and 50.7±8.1 ng/mg protein, respectively) (Figure 2b).

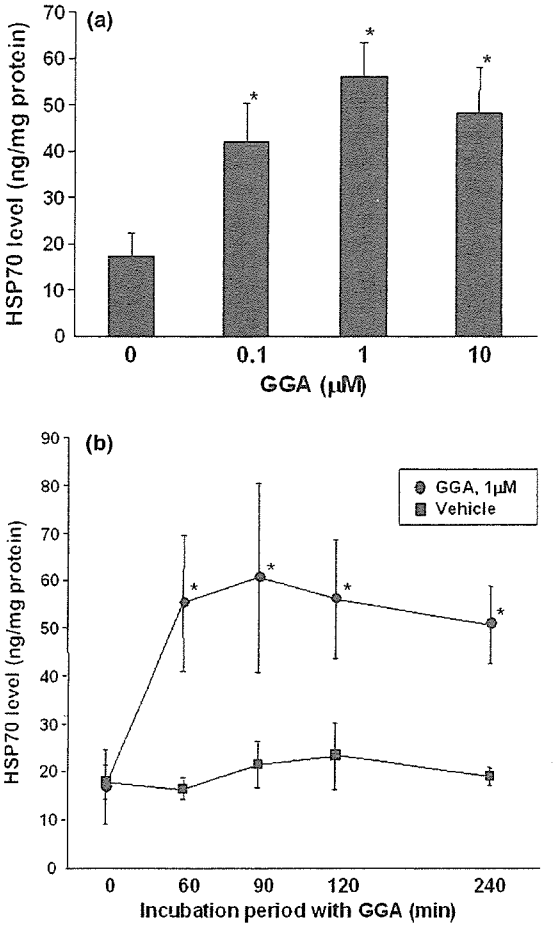

Figure 2. Induction of HSP70 by geranylgeranylacetone (GGA) in rat intestinal cells. (a) Dose–response analysis of HSP70 induction by GGA. Intestinal epithelial cell (IEC)-18 cells were incubated with the indicated concentrations (0.1, 1, 10 μM) of GGA for 90 min. (b) Time-course analysis of HSP70 induction by GGA. Cells were incubated with 1 μM GGA for 60, 90, 120 and 240 min. Endogenous HSP70 levels in cells were measured by ELISA. Values are means±SD: N=5; *$p < 0.01$ versus vehicle-treated cells.

Quercetin inhibits induction of HSP70 and cell protection by GGA against oxidative stress in IEC-18 cells

To clarify the effects of HSP70 induced by GGA on cell injury in IEC-18 cells, we next examined the effects of quercetin, which inhibits HSP70 synthesis, on cell protection by GGA in IEC-18 cells. Pretreatment with 200 μM quercetin for 2 h significantly reduced OD values in IEC-18 cells treated with 1 μM GGA compared with the cells without quercetin treatment when cells were exposed to NH_2Cl ($p < 0.01$; 0.38±0.13 and 0.68±0.19, respectively) (Figure 3), whereas the difference in OD in the quercetin-treated IEC-18 cells without NH_2Cl was minimal compared with non-treated cells (0.83±0.12 and 0.88±0.12, respectively) (Figure 3). In HSP70 expression, 200 μM quercetin completely diminished elevation of HSP70 by 1 μM GGA for 90 min in the IEC-18 cells compared with GGA-treated cells without quercetin treatment ($p < 0.01$; 21.2±4.0 and 48.7±7.2 ng/mg protein, respectively) (Figure 4). On the other hand, quercetin failed to change the baseline levels of HSP70 in the IEC-18 cells without GGA treatment (15.7±4.2 and 17.2±1.2 ng/mg protein, respectively) (Figure 4).

Discussion

GGA has been reported to prevent gastric ulcer and erosion in animal experimental models induced by chemicals [8,11,22]. GGA plays a cytoprotective role in gastric mucosal damage caused by

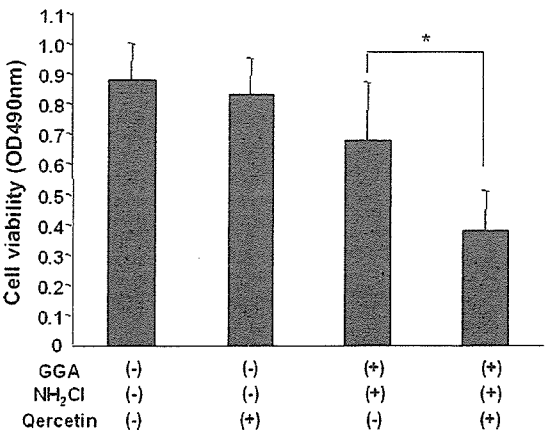

Figure 3. Effects of quercetin on the protection of intestinal epithelial cell (IEC)-18 cells by geranylgeranylacetone (GGA) from NH_2Cl. IEC-18 cells were preincubated with 200 μM quercetin for 2 h; 1 μM GGA or vehicle was then added to the medium for 90 min. After GGA treatment, NH_2Cl was directly added to the culture medium to a final concentration of 0.3 mM and cells were incubated for 1 h. Cell viability was determined by XTT assay. Values are means±SD. N=5; *$p < 0.01$.

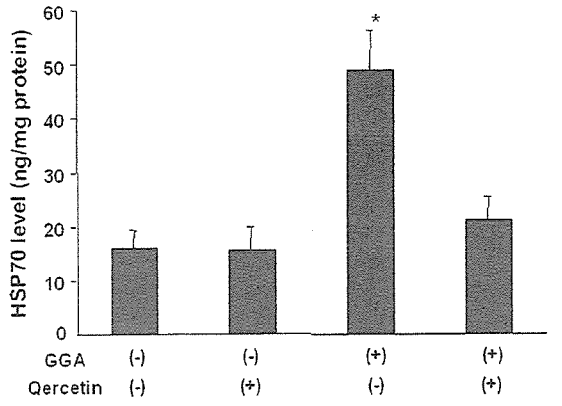

Figure 4. Effects of quercetin on induction of HSP70 by geranylgeranylacetone (GGA) in intestinal epithelial cell (IEC)-18 cells. Cells were preincubated with 200 μM quercetin for 2 h; 1 μM GGA was then added to the medium for 90 min. Endogenous HSP70 levels in the cells were measured by ELISA. Values are means ± SD. N = 5; *p < 0.01 versus non-treated cells.

non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin [8,9,23]. In other tissues, GGA has been shown to protect tissues such as heart, liver, and brain from ischemic, chemical, and surgical injuries [15,16,24]. In the present study, consistent with these findings, we demonstrated for the first time that GGA protects a rat intestinal cell line, IEC-18 cells, from oxidative injury. Our findings suggest that GGA plays an important role in the protection of intestinal epithelial cells from damage and injury caused by oxidants and other pathogens.

Recent studies have clarified that one of the defense mechanisms of GGA in cells and tissues against stress is primarily to up-regulate the expression of endogenous HSP70, which plays an important role in cell protection [5,14]. GGA suppresses apoptosis of cultured rat hepatocytes caused by hydrogen peroxide and ethanol via enhancement of HSP70 expression [25]. *In vitro*, GGA has been reported rapidly to induce HSP70. Up-regulation of HSP70 by GGA in hepatocytes is seen within 1 h of GGA treatment and reaches a peak 2 h after treatment with GGA [25]. Hirakawa et al. have shown the mechanism of rapid induction of HSP70 in rat gastric mucosa [14]. Stress promotes and enhances heat shock factor (HSF)-1, and transduction of HSF-1 activates synthesis of HSP mRNA. Without processing of HSP70 mRNA, HSP70 is rapidly synthesized in the cells. Consistent with previous reports, we found that the protective effects of GGA could be observed at 60 min after GGA treatment and sustained until 240 min after GGA treatment. At 60 min, the HSP70 levels increased rapidly after GGA treatment. Although the precise mechanism of rapid induction of HSP70 by GGA remains unknown, it is conceivable that transduction of HSF-1 activates synthesis of HSP mRNA in IEC cells treated with GGA, as suggested [14].

Overexpression of HSP70 is important for tissue protection against oxidative and ischemic stresses [26,27]. In IEC-18 cells, HSP70 enhanced by non-lethal heat stress, glutamine and dexamethazone critically protects cells against NH_2Cl and lethal heat stress [6,7,20]. Consistent with these findings, we found in this study that expression of endogenous HSP70 in IEC-18 cells treated with GGA was increased more than 2-fold compared with the vehicle-treated cells. Quercetin, a flavenoid, is known to inhibit HSP70 synthesis. It has been reported that quercetin diminishes the induction of HSP70 expression and reduces cell viability against oxidative stress and lethal heat shock in cells such as colon carcinoma and IEC-18 cells [7,21]. In this study, we demonstrated that quercetin completely diminishes the protective effects of GGA upon oxidative injury in IEC-18 cells. It is considered that quercetin suppresses the effects of GGA upon induction of HSP70 and cytoprotection from oxidative stress in IEC-18 cells. These findings suggest that GGA protects IEC-18 cells against oxidative injury, primarily through the protective effects of HSP70.

In conclusion, we have shown for the first time that GGA protects rat IECs against oxidative stress. The cell-protective effects of GGA are considered to be dependent on overexpression of HSP70 by GGA. Our study provides novel evidence that GGA is useful for treatment of intestinal injuries, including inflammatory bowel disease and other types of colitis.

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Resistance to experimental colitis depends on cytoprotective heat shock proteins in macrophage migration inhibitory factor null mice

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Received 15 July 2006; received in revised form 3 September 2006; accepted 3 September 2006
Available online 26 September 2006

Abstract

Macrophage migration inhibitory factor plays an important role in inflammatory diseases. We investigated the role of macrophage migration inhibitory factor (MIF) in the development of dextran sulfate sodium (DSS)-induced colitis using MIF null ($^{-/-}$) mice. MIF$^{-/-}$ mice given 3% DSS showed no clinical and histological feature of colitis in contrast to wild-type (WT) mice. Lack of MIF suppressed the up-regulation of TNF-α and IFN-γ as Th1-derived cytokines, and increased the level of IL-4 as Th2-derived cytokine in the colon tissues. Moreover, we found that the expressions of heat shock protein (HSP)40 and HSP70 were markedly up-regulated in the colon of MIF$^{-/-}$ mice in response to DSS compared with WT mice. Additionally, quercetin, an inhibitor of HSP synthesis, inhibited the up-regulation of HSP40 and 70 expressions and developed DSS-induced colitis in MIF$^{-/-}$ mice. Our findings in this study provide more information in the role of MIF in colitis.

Keywords: Colitis; Cytokines; Heat shock protein; Macrophage migration inhibitory factor

1. Introduction

Inflammatory bowel diseases (IBDs) such as ulcerative colitis and Crohn's disease are characterized by chronically relapsing inflammation of the bowel of unknown etiology [1]. Although the mechanisms of the inflammation and immune responses in IBD are not yet fully understood, it has been suggested that various inflammatory mediators such as the tumor necrosis factor (TNF)-α and macrophage migration inhibitory factor (MIF) are involved in their pathogenesis and exacerbation [2–7]. On the other hand, experimental animal colitis models are used for investigation on the mechanism of the development of colitis. In particular, dextran sulfate sodium (DSS)-induced colitis model has been widely deployed because of its reliability and simplicity among several experimental models mimicking human IBD [8,9].

MIF was originally discovered as a soluble factor associated with the mechanism of delayed-type hypersensitivity [10,11], but today is widely accepted to be a pluripotent cytokine involved in a broad-spectrum of physiological and pathological events beyond the immune system [12]. As a new biological aspect, it has been reported that MIF genetic polymorphism is associated with the development of juvenile-onset systemic idiopathic arthritis [13,14]. MIF is expressed in a variety of cells, including

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doi:10.1016/j.imlet.2006.09.002

lymphocytes and macrophages, and exerts an array of biological functions relevant to not only immune responsiveness [15–18], but also tumorigenesis [19]. On the other hand, MIF secretion is associated with a multitude of diseases in humans, and it is of note that MIF is the key mediator for the expression of inflammatory cytokines, particularly TNF-α.

Recently, we have demonstrated that an anti-MIF antibody is remarkably effective in preventing DSS-induced colitis through the suppression of proinflammatory cytokines such as TNF-α [5–7]. However, the precise molecular-basis of the mechanism by which this works remains to be elucidated. The aim of this study was to investigate the role of MIF in the inflammatory states in DSS-induced colitis using MIF null ($^{-/-}$) mice. Interestingly, we found that resistance to DSS-induced colitis depended on the expression and function of some HSPs in MIF$^{-/-}$ mice. Our present studies provide more information in the role of MIF in colitis.

2. Materials and methods

2.1. Materials

DSS (molecular weight, 40 kDa) from ICN Biomedicals (Costa Mesa, CA); ELISA development kits for mouse TNF-α, IFN-γ and IL-4 from Genzyme Techne (Minneapolis, MN); quercetin from Sigma (St. Louis, MO); Micro BCA protein assay reagent kit from Pierce (Rockford, IL); Vectastain ABC kit from Vector Laboratories (Burlingame, CA); and all antibodies against HSPs, including HSP25, HSP32, HSP40, HSP70 and HSP90, from Stressgen (Victoria, BC, Canada). All other chemicals were of reagent grade.

2.2. Animals

By targeted disruption of the MIF gene, a mouse strain (bred onto a BALB/c background) deficient in MIF was established, and maintained under specific pathogen-free conditions [20]. All animal procedures were conducted according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol. The 8–9-week-old male mice were used in each experiment.

2.3. Induction and general assessment of experimental colitis

To induce colitis, we administered 3.0% DSS in distilled water to BALB/c mice ad libitum. Mice were weighed and visually inspected for anal bleeding and diarrhea. Animals were euthanized by intraperitoneal injection of thiopental at day 7 post-DSS unless otherwise specifically indicated. The severity of colitis was also evaluated by assessment of colon length and histological examination.

2.4. Pathology

The tissues of the colon were longitudinally opened, fixed with 10% neutral buffered formalin, and embedded in paraffin. After deparaffinizing of thin tissue sections on glass slides, the samples were stained with H&E. For histological evaluation of the tissue damage, areas of inflammatory lesions were microscopically evaluated and quantitated as reported previously [5]. Briefly, the tissue damage was categorized into six grades: grade 0, normal mucosa; grade 1, infiltration of inflammatory cells; grade 2, shortening of the crypt by less than half; grade 3, shortening of the crypt by more than half; grade 4, crypt loss, and grade 5, destruction of epithelial cells (ulceration and erosion). In addition, we evaluated the extent of inflammatory lesions. The extent of the lesions in the total colon was classified into six grades: grade 0, 0%; grade 1, 1–20%; grade 2, 21–40%; grade 3, 41–60%; grade 4, 61–80%; grade 5, 81–100%. In grading of histological scores, two pathologists evaluated each section in a blind fashion.

2.5. Tissue cytokine measurement

For measurement of mouse TNF-α, IFN-γ and IL-4, their contents in colon tissues were measured using ELISA kits specific for each cytokine as described previously [5].

2.6. Western blot analysis

The samples of colon tissues in PBS with protease inhibitor cocktail (Sigma, St. Louis, MO) were disrupted with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Equal amounts of homogenates were dissolved in 20 μL of Tris–HCl, 50 mM (pH 6.8), containing 2-mercaptoethanol (1%), SDS (2%), glycerol (20%), and bromophenol blue (0.04%), and the samples were heated at 100 °C for 5 min. They were then subjected to SDS-PAGE and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked with 1% nonfat dry milk in PBS, probed with HSP25, 32, 40, 70 and 90 antibodies, and reacted with a goat anti-rabbit IgG antibody coupled with horse radish peroxidase. The resultant complexes were processed for the detection system according to the manufacturer's protocol. The protein concentrations of the cell homogenates were quantified using a Micro BCA protein assay reagent kit.

2.7. Treatment with quercetin as an inhibitor of HSP synthesis in mice

Quercetin is used as an inhibitor of HSP synthesis in vitro and in vivo [21,22]. In this experiment, to interrupt the synthesis of HSP, we intraperitoneally injected MIF$^{-/-}$ mice with 200 mg/kg of quercetin in 0.2 mL of distilled water 2 h before and once a day for 6 days. Mice without DSS treatment were administrated with water for 7 days. At day 7, clinical features and histology are evaluated, and Western blot analysis for HSP was performed in the samples of colon.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (S.D.). The results of change in body weight, histological scores and

Table 1
Clinical signs and colon length

	Diarrhea	Rectal bleeding	Occult blood in feces	Colon length (cm)
Non-treated WT	0/10*	0/10*	0/10*	8.8 ± 0.2**
WT + DSS	10/10	10/10	10/10	6.0 ± 0.4
MIF$^{-/-}$ + DSS	0/10*	0/10*	0/10*	8.3 ± 0.2**

Wild-type and MIF null mice were fed 3.0% DSS for 7 days ($n = 10$, each group). Values are means ± S.D. Data on the consistency of diarrhea, rectal bleeding and occult blood in feces were statistically analyzed by Fisher's exact test, and data on the colon length were analyzed using ANOVA and Student's t-test. *$p < 0.01$ and **$p < 0.001$: statistically significant compared with DSS-treated WT mice. WT: wild-type mice, MIF$^{-/-}$: MIF null mice, DSS: dextran sulfate sodium.

the levels of cytokines were statistically analyzed using one-way ANOVA and pos hoc test using Student's unpaired t-test, and the data of clinical findings in diarrhea, rectal bleeding and occult blood test in feces were analyzed by Fisher's exact test (StatView, SAS Institute, Cary, NC).

3. Results

3.1. Clinical feature and histology

A 3.0% DSS solution clinically and histologically induced severe colitis in all wild-type (WT) mice. Surprisingly, no sign of the clinical symptoms regarding colitis was observed in MIF$^{-/-}$ mice at day 7 post-DSS treatment (Table 1). The colon length in MIF$^{-/-}$ mice was significantly longer than that of DSS-treated WT mice. Body weight loss was minimal in the MIF$^{-/-}$ mice in contrast to DSS-treated WT mice (Fig. 1). We assessed histological changes in the colon to confirm the resistance of MIF$^{-/-}$ mice against the challenge by DSS. Marked infiltration of mononuclear cells was observed at day 7 post-DSS with crypt loss and destruction of epithelial cells in WT mice (Fig. 2B), in comparison with the non-treated controls (Fig. 2A). Consistent

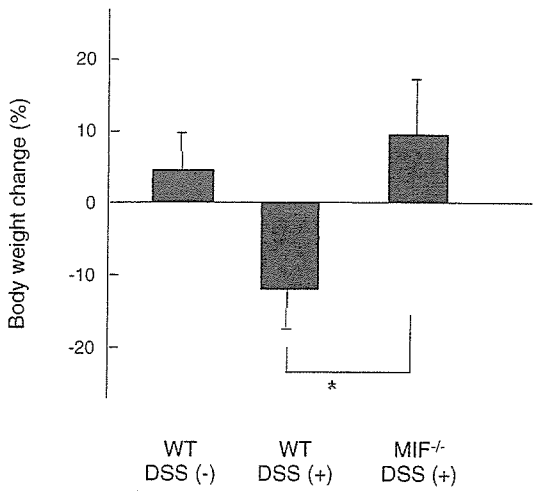

Fig. 1. Body weight changes of mice in DSS-induced colitis ($n = 10$ in each group). We administered 3.0% DSS in distilled water to wild-type (WT) and MIF null ($^{-/-}$) mice for 7 days. Mice were weighed 7 days post-DSS. Percent in body weight change was calculated in comparison with body weight at day 0 (100%), *$p < 0.01$ as compared with non-treated WT mice.

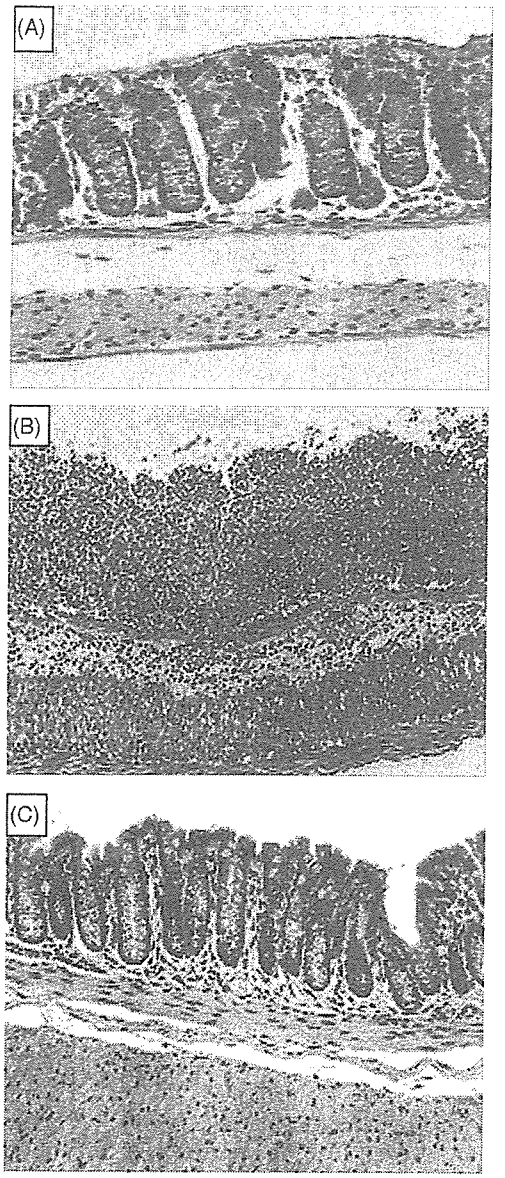

Fig. 2. Histological sections of the colons in WT and MIF$^{-/-}$ mice. The tissue sections from mice treated with 3.0% DSS or water for 7 days were stained with H&E. The sections of water-treated WT mice (A), 7 days-DSS-treated WT mice (B), 7 day-DSS treated MIF$^{-/-}$ mice (C). Representative pictures are shown. Original magnification 100×.

with clinical signs, the degree of tissue damage in MIF$^{-/-}$ mice was minimal (Fig. 2C). The histological scores in tissue damage and extent of lesions were significantly ($p < 0.01$) lower in the colon of MIF$^{-/-}$ mice than that of WT mice (tissue damage, 0.1 ± 0.1 and 4.5 ± 0.2; extent of lesion, 0.1 ± 0.1 and 4.0 ± 2.2, respectively, $n = 10$ in each group).

3.2. Cytokine contents in colon tissues

We evaluated the contents of TNF-α, IFN-γ and IL-4 in the colon of mice. Both the TNF-α and IFN-γ levels were significantly increased in WT mice with DSS colitis, whereas these cytokines were not increased in the colon of MIF$^{-/-}$ mice even when mice were treated with DSS (Fig. 3A and B). In addi-

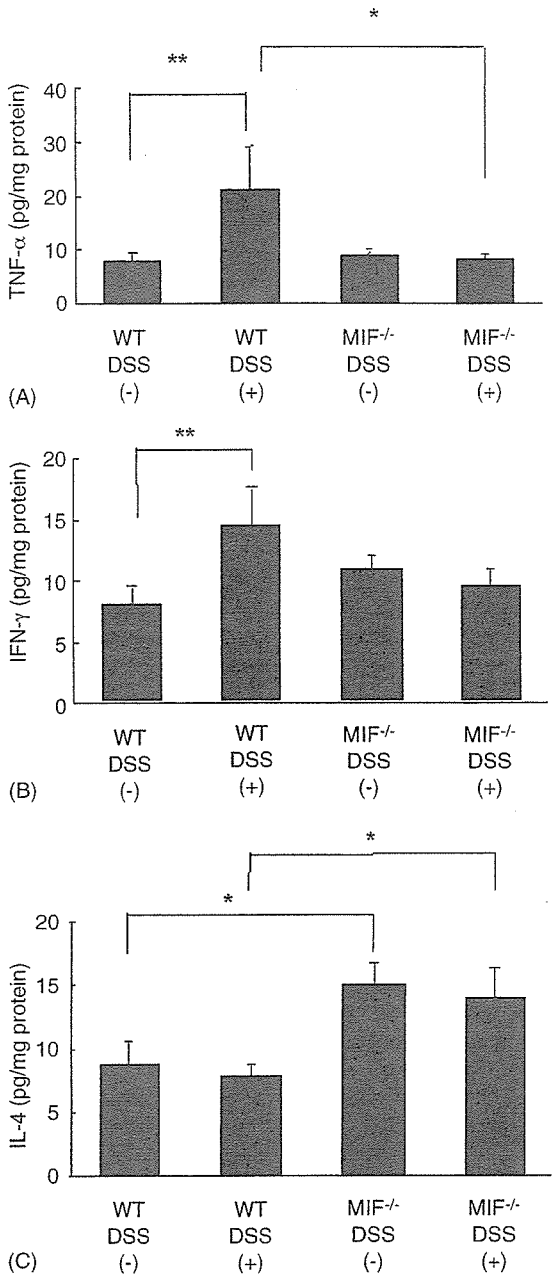

Fig. 3. (A–C) Th1/Th2 cytokine contents in the colon of DSS-induced colitis mice. TNF-α and IFN-γ (Th2-type cytokines) and IL-4 (Th2-type cytokine) in the colons of MIF$^{-/-}$ mice ($n=5$) and WT mice ($n=5$) before and 7 days after DSS treatment were measured by ELISA. $^{*}p<0.01$, $^{**}p<0.05$.

tion, the IL-4 level was significantly increased in MIF$^{-/-}$ mice without DSS treatment, and the level was essentially unchanged when mice were given DSS for 7 days. In contrast to MIF$^{-/-}$ mice, the IL-4 level in the colon of WT mice were lower than that of MIF$^{-/-}$ mice, and this level was unchanged in WT mice with DSS colitis (Fig. 3C).

3.3. Expression of HSP in colon tissues

We investigated the expression of HSP in the colon of MIF$^{-/-}$ mice. As for the HSP family protein, we found that the HSP40 and HSP70 expressions in the colon tissues were exceptionally

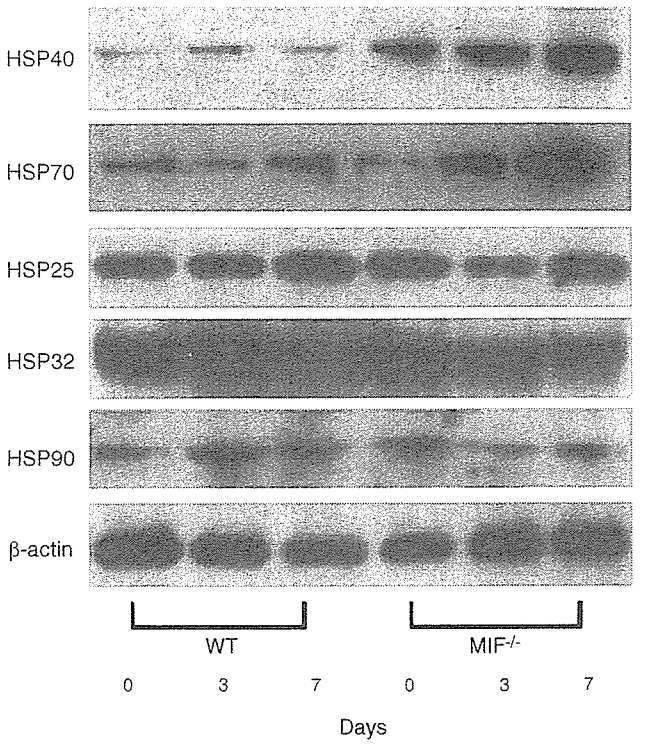

Fig. 4. Expressions of HSPs in the colon in WT and MIF$^{-/-}$ mice following DSS treatment. Total colon homogenates collected before and post-DSS at days 3 and 7 were prepared from WT and MIF$^{-/-}$ mice. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the expressions of HSP40, HSP70, HSP25, HSP32, and HSP90 using antibodies specific for each HSP. Representative data are shown. Similar results were obtained from three independent experiments.

up-regulated at days 3 and 7 post-DSS, as assessed by Western blot analysis (Fig. 4). However, the expression of other HSP family proteins, including HSP25, HSP32, and HSP90, was essentially unchanged (Fig. 4).

3.4. Quercetin inhibits induction of HSPs and develops DSS-induced colitis in MIF$^{-/-}$ mice

To investigate the role of enhanced expression of HSP in the protection of DSS-induced colitis in MIF$^{-/-}$ mice, we test the effect of quercetin on colitis in MIF$^{-/-}$ mice treated with DSS. All MIF$^{-/-}$ mice did not show any abnormal appearances and histological findings of colitis when mice were treated with only quercetin (Table 2). In contrast, MIF$^{-/-}$ mice presented severe diarrhea, bloody stool and shortening of colon length when mice were treated with quercetin and DSS for 7 days (Table 2). Histological scores in tissue damage and extent of lesion were significantly higher ($p<0.001$) in MIF$^{-/-}$ mice treated with quercetin and DSS than those of MIF$^{-/-}$ mice treated with DSS only (tissue damage, 3.8 ± 0.4 and 0.0 ± 0.0; extent of lesion, 3.4 ± 0.2 and 0.0 ± 0.0, respectively, $n=10$ in each group). In addition, treatment with quercetin did not up-regulate the expressions of HSP40 and HSP70 in the colon of MIF$^{-/-}$ mice given DSS for 7 days (Fig. 5). On the other hand, quercetin did not alter the expressions of these HSPs in the colon of MIF$^{-/-}$ mice without DSS treatment (Fig. 5).

Table 2
Clinical signs and colon length in mice treated with quercetin and/or DSS

	Diarrhea	Rectal bleeding	Occult blood in feces	Colon length (cm)
$MIF^{-/-}$ + Q	0/10	0/10	0/10	8.9 ± 0.2
$MIF^{-/-}$ + DSS	0/10	0/10	0/10	8.8 ± 0.1
$MIF^{-/-}$ + Q and DSS	9/10*	7/10*	10/10*	6.9 ± 0.4*

$MIF^{-/-}$ mice were fed 3.0% DSS for 7 days ($n = 10$, each group). Values are means ± S.D. Data on the consistency of diarrhea, rectal bleeding and occult blood in feces were statistically analyzed by Fisher's exact test, and data on the colon length were analyzed using ANOVA and Student's t-test. *$p < 0.01$: statistically significant compared with DSS-induced $MIF^{-/-}$ mice. $MIF^{-/-}$: MIF null mice; DSS: dextran sulfate sodium; Q: quercetin.

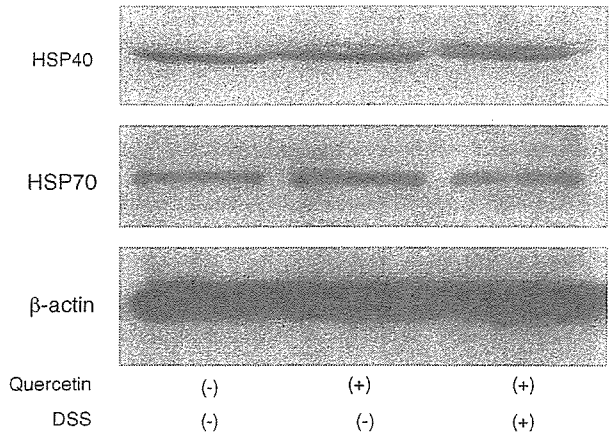

Fig. 5. Expressions of HSP40 and HSP70 in the colon in $MIF^{-/-}$ mice given quercetin and/or DSS at 7 day. Western blot analysis for HSP was performed in the samples of total colon homogenates. Representative data are shown. Similar results were obtained from three independent experiments.

4. Discussion

MIF is essentially involved in several inflammatory disorders, including septic shock [17,23], inflammatory lung diseases [24], and autoimmune diseases [25]. Recently, de Jong et al. and we have focused on the pathological role of MIF in colitis, following the revelation of its pathogenic role in disease [4,5]. In this study, we demonstrated that $MIF^{-/-}$ mice did not show any features of DSS-induced colitis. This finding was consistent with our previous finding that an anti-MIF antibody effectively suppressed inflammatory processes in DSS-induced colitis in mice [5]. To date, clinical evidence suggesting the pathogenic role of MIF in IBD has been reported [4,5,26]. These facts including our present results provide the strong evidence that MIF plays a critical role in the development of intestinal inflammation.

MIF is known to act as a proinflammatory cytokine; however, its precise actions with regard to the Th1/Th2 balance have not fully understood in colitis. Our previous study that Th1-type cytokines (TNF-α and IFN-γ) are up-regulated by DSS treatment suggests that MIF contributes to the production of these cytokines [5]. Consistent with our previous data, the contents of Th1-type cytokines (TNF-α and IFN-γ) in the colon of $MIF^{-/-}$ mice were minimally changed by DSS treatment. Interestingly Th2-type cytokine (IL-4) was significantly increased in $MIF^{-/-}$ mice, and this level was essentially unchanged by DSS treatment compared with WT mice with or without DSS-induced colitis. Recent studies by Bacher et al. and Nishihira have shown the essential role of MIF for T cell activation and cytokine production [18,19] Based on these reports and our current results, it is conceivable that MIF, in colon, may activate T cell function and induce the production of Th1-type cytokines, promoting inflammation and immune responses, whereas this protein may regulate Th2-type cytokine production in a negative manner.

To investigate another reason why $MIF^{-/-}$ mice were resistant to DSS-induced colitis, we focused on the expression of HSP in colon because of its anti-inflammatory and cell-protective properties. Bourdi et al. have reported that the induction of several HSPs are greater in $MIF^{-/-}$ mice than in WT mice following drug-induced liver injury, suggesting that the proinflammatory/protoxicant properties of MIF occur through its down-regulatory effects on HSP expression [27]. In addition, induction of HSP prevents apoptosis by regulating intracellular intermediates intimately involved in apoptotic signaling.

Previous studies have shown that MIF has an anti-apoptotic function [28–30]. Because of its role of MIF, It is expected that complete deletion of MIF promotes the process of apoptosis in various cells and tissues. In vitro, there are observations with the anti-apoptotic effects of MIF [28–30]; however, several studies have demonstrated that $MIF^{-/-}$ mice are resistant to tissue damage induced by chemicals such as colitis and hepatitis [4,27]. The results of these studies in vivo seem to be inconsistent with the anti-apoptotic effects of MIF in vitro. In this study, consistent with the report by Bourdi et al., we also demonstrated that some HSPs, which have an anti-apoptotic property, were predominantly induced in vivo under conditions of lacking MIF and that these HSPs contributed to prevention from DSS-induced colitis. Besides the activation of immune response by DSS treatment, DSS is known to be toxic and directly damage the epithelial cells in colon [31]. Thus, our results provide the hypothesis that the elevated HSP expression in the colon of $MIF^{-/-}$ mice is an important responsive mechanism to counter apoptotic events after DSS.

Up-regulation of HSP protects host cells from physical stresses, infection and inflammation. Thus, HSP induction has been suggested to be a key anti-inflammatory stimulus, and is widely accepted to be a critical protective mechanism against physiologic and environmental stressors [32]. HSP70 is a part of members in HSP70 family, which contains a number of highly related protein isoforms ranging in size from 66 to 78 kDa. Members of the HSP70 family are molecular chaperones, which are involved in many cellular functions, such as protein folding, transport, maturation and degradation. In heat-shocked cells,