ACKNOWLEDGMENTS

THIS WORK WAS supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture (no. 17015016 to H. M.) and from the Ministry of Health, Labor and Welfare of Japan (to H. M.).

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Chemico-Biological Interactions 184 (2010) 423–430

Contents lists available at ScienceDirect

Chemico-Biological Interactions

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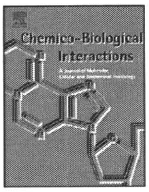

Genetic ablation of *Tnfα* demonstrates no detectable suppressive effect on inflammation-related mouse colon tumorigenesis

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ARTICLE INFO

Article history:
Received 16 December 2009
Received in revised form 4 January 2010
Accepted 6 January 2010
Available online 14 January 2010

Keywords:
Tnfα
Inflammation-related colorectal cancer
Mouse

ABSTRACT

Colorectal cancer (CRC) is one of the most serious complications of inflammatory bowel disease. Tumor necrosis factor-α (Tnfα) is a major mediator of inflammation and there is increasing evidence that Tnfα/Tnf-receptor-1 (Tnfr1) signaling may act as an endogenous tumor promoter for colon carcinogenesis. In fact, a previous study revealed that mice lacking *Tnfr1* develop significantly fewer colonic tumors in the inflammation-related CRC model. In addition, antibodies against Tnfα have been shown to inhibit the development of inflammation-related CRC. In the present study, *Apc* Min/+; *Tnfα* −/− mice were treated with 2% dextran sodium sulfate (DSS) and the tumor development was compared with *Apc* Min/+; *Tnfα* +/+ control mice in order to investigate the role of Tnfα by itself in the inflammation-related CRC. Surprisingly, there were no detectable differences in either the severity of colonic inflammation or the expression of DSS-induced chemokines and cytokines (*Ccl2*, *Cxcl1*, *Tnfβ*, *Il1β*, *Il6*, and *Cox-2*) that relate to the colonic inflammation and tumorigenesis between these two groups. Furthermore, the genetic ablation of *Tnfα* did not suppress the colon tumorigenesis in comparison to the wild-type mice. Our observations suggest that intricate inflammatory responses promote the inflammation-related mouse colon tumorigenesis.

1. Introduction

The link between carcinogenesis and chronic inflammation has been recognized for certain types of cancer, including colorectal cancer (CRC) [1]. CRC is one of the serious complications of inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease [1,2]. Although previous studies have demonstrated that the link between inflammation and CRC offers a possible strategy to prevent CRC, the underlying molecular processes involved in this interaction still remain poorly understood. Tumor necrosis factor-α (Tnfα) is a key cytokine involved in inflammation, immunity and cellular organization [3]. It was first isolated from the serum of mice infected with *Bacillus-Calmette–Guerin* treated with endotoxin, and shown to replicate the ability of endotoxin to induce haemorrhagic tumor necrosis [4]. Accordingly, it was originally utilized for the treatment of patients with advanced solid tumors [5]. In contrast, recent evidence indicates that Tnfα may act as an endogenous tumor promoter in several tumor tissues. Direct evidence for the involvement of Tnfα in malignancy came from observations that a genetic disruption of the *Tnfα* gene could significantly attenuate chemically induced skin tumor formation [6–8]. In addition, *Tnf-receptor-1* (*Tnfr1*) deficient mice had reduced oval cell (the putative hepatic stem cell) proliferation during the pre-neoplastic phase of liver carcinogenesis, correlating with fewer liver tumors than wild-type mice [9]. It has therefore been suggested that the Tnfα/Tnfr1 signaling pathway may play an important role in tumor promotion.

The predominant expression of Tnfα in colorectal cancer is observed within tumor-associated macrophages [10]. A previous study reported that Tnfα and Tnfr1 protein are expressed mainly in the infiltrating cells, such as macrophages and neutrophils which are derived from myeloid cells in inflamed colon tissue [11]. These infiltrating cells also express Cox-2 protein which is often up-regulated in colon carcinoma tissues and functionally promotes intestinal tumorigenesis [12]. Furthermore, Greten et al. reported that depleting *Ikkβ* in myeloid cells reduced the expression of pro-

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Abbreviations: Tnfα, tumor necrosis factor-α; IBD, inflammatory bowel disease; Tnfr1, tumor necrosis factor-receptor-1; CRC, colorectal cancer; DSS, dextran sodium sulfate; Apc, adenomatous polyposis coli; Min, multiple intestinal neoplasia.

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doi:10.1016/j.cbi.2010.01.014

inflammatory factor genes encoding Tnfα, Il1β, Il6, Kc, Cox-2 and Mmp-9 in the colon, followed by a suppression of colorectal tumor development in inflammation-related colon tumorigenesis [13].

It is noteworthy that the absence of *Tnfr*1 significantly reduces the DSS-induced infiltration of Cox-2-expressing inflammatory cells, thus leading to a reduced incidence of colonic tumors [11]. Moreover, Tnfα can significantly increase Nf-kb activation in various cell types after binding to either Tnfr1 or Tnf-receptor-2 (Tnfr2) [14]. Therefore, it has been thought that endogenous Tnfα may activate Nf-kb signaling in inflammatory cells by interacting with Tnfr1 in an autocrine/paracrine manner and activated Nf-kb increases the expression of pro-inflammatory factors, which could thus lead to the promotion of colorectal tumorigenesis. Given the fact that Tnfr1 signaling is associated with tumorigenesis in the colon, Tnfα itself might be a therapeutic target for the inflammation-related mouse colon tumorigenesis. Indeed, previous studies demonstrated that antibodies against Tnfα inhibit the development of inflammation-related CRC [15].

Several animal models of experimental colitis have been developed for investigating the pathogenesis of IBD and IBD-related CRC and these are often used to evaluate new treatments for IBD [16]. Chemically induced models of intestinal inflammation, such as the dextran sodium sulfate (DSS)-induced model and the trinitrobenzene sulfonic acid (TNBS)-induced model, are the most generally used IBD animal models because the onset of inflammation is immediate and the procedure is relatively straightforward [17,18]. In contrast to the involvement of CD4+ T cells in TNBS-induced colitis, macrophages have been shown to play a central role in DSS-induced colitis [18,19]. Importantly, once *Apc* Min/+ mice, which harbor a germ line mutation in the *Apc* gene, are exposed to DSS, colitis markedly accelerates the development of dysplasia and cancer in the colon of *Apc* Min/+ mice [20]. Therefore, DSS-treated *Apc* Min/+ mouse can be useful for the investigation of inflammation-related colorectal tumorigenesis. The current study examined the effects of the genetic ablation of *Tnfα* on colon tumorigenesis using *Apc* Min/+; *Tnfα* −/− compound mutant mice exposed to DSS in order to determine whether Tnfα by itself could be a target for the prevention/treatment of inflammation-related colon tumorigenesis.

2. Materials and methods

2.1. Animals and diets

Apc Min/+ mice in the C57BL/6J background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). *Tnfα* −/− mice were maintained in C57BL/6J background [21]. We confirmed that the cell viability of splenocytes from *Tnfα* −/− mice is reduced after the stimulation with phorbol 12-myristate 13-acetate (PMA; 80 nM) and ionomycin (1 μM) when compared with that from *Tnfα* +/+ mice, thus suggesting the distinguishable responses against inflammatory stimuli between *Tnfα* +/+ and *Tnfα* −/− mice. Compound mutant *Apc* Min/+; *Tnfα* −/− mice were generated by breeding *Apc* Min/+; *Tnfα* +/− males to *Apc* +/+; *Tnfα* +/− females. These mice were maintained on a C57BL/6J genetic background to avoid potential strain differences in phenotype. All mice were bred and maintained in a specific pathogen-free animal facility under standard 12:12 h light:dark cycle and fed on a basal diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan), and water *ad libitum* until the termination of the study.

2.2. Experimental procedures

DSS with a molecular weight of 36,000–50,000 (Wako, Osaka, Japan) was dissolved in distilled water at a concentration of 2% (w/v). Initially, 26 *Apc* Min/+; *Tnfα* +/+ mice (14 males and 12 females) and 25 *Apc* Min/+; *Tnfα* −/− mice (14 males and 11 females) were used for the macroscopic and histological study. The animals of these cohorts were given 2% (w/v) DSS in drinking water for 1 week, starting at 5 weeks of age, according to the protocol described in previous report [20]. The DSS-exposed *Apc* Min/+; *Tnfα* +/+ mice and *Apc* Min/+; *Tnfα* −/− mice were then sacrificed at 10 weeks of age for both the macroscopic inspection and histological analysis (Fig. 1a). Next, 20 male *Tnfα* +/+ mice and 19 male *Tnfα* −/− mice were treated with 2% (w/v) DSS in drinking water for 5 days, starting at 5 weeks of age, and then were sacrificed at day 0 (control, five mice in each group), day 10 (the acute phase of colonic inflammation, seven mice in each group) and day 30 (the chronic phase of colon inflammation, eight mice in *Tnfα* +/+ and seven mice in *Tnfα* −/−) after the exposure of DSS in order to compare the induction of pro-inflammatory factors in the presence or absence of *Tnfα* (Fig. 1b). Trinitrobenzene sulfonic acid (TNBS) was also used for another model of colonic inflammation. TNBS colitis was induced in five male *Tnfα* +/+ and six male *Tnfα* −/− mice according to the method of Wirtz et al. [18] with minor modifications. After the TNBS presensitization, the mice were lightly anesthetized, and an infant feeding catheter (3.5 Fr) was then carefully inserted into the colon such that the tip was 4 cm proximal to the anus. Five percent (w/v) in H_2O TNBS solution dissolved in the same volume of 100% ethanol was then slowly administered into the colon lumen through the catheter. The total injection volume was 100 μl in both groups, thus allowing TNBS to reach the entire colon. After the above administration, the mice were kept upside down while being held by their tails for 60 s and then were returned to their cages. All mice were then sacrificed at day 12 for both macroscopic inspection and histological analysis. At autopsy in each group of mice, their large bowel was flushed with saline, and then was excised. The large bowel from the ileocecal junction to the anal verge was measured, cut open longitudinally along the main axis, and then washed with saline. The total tumor number, tumor localization and the size of each tumor were recorded. The tumor volume was calculated as length × width × width × 0.526 [22]. After macroscopic inspection, it was rolled like a "*Swiss roll*" and then fixed overnight in 10% buffered formalin. Paraffin-embedded sections were made using routine procedures.

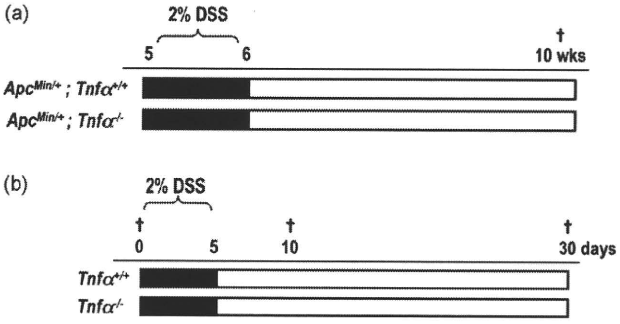

Fig. 1. Experimental protocols for this study. (a) Experimental design to investigate the role of Tnfα in DSS-induced colon tumorigenesis. (■) Basal diet and 2% DSS in drinking water, (□) basal diet and tap water, (✝) sacrifice. (b) Experimental design to compare the induction of pro-inflammatory factors in the presence or absence of *Tnfα*. Day 0, day 10 and day 30 represent control, the acute phase of colonic inflammation and the chronic phase of colon inflammation, respectively. (■) Basal diet and 2% DSS in drinking water, (□) basal diet and tap water, (✝) sacrifice.

2.3. Histological inflammation score

The histopathological alterations of the colon were examined on hematoxylin and eosin (H&E) stained sections and colon inflammation was scored according to the following morphological criteria as described previously [23]. Grade 0, normal colon mucosa; Grade 1, shortening and loss of the basal one-third of the actual crypts with mild inflammation and edema in the mucosa; Grade 2, loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa; Grade 3, loss of all crypts with severe inflammation in the mucosa, but with retention of the surface epithelium; and Grade 4, loss of all crypts and surface epithelium with severe inflammation in the mucosa.

2.4. *Immunohistochemical analysis*

The avidin–biotin peroxidase complex technique was used for immunohistochemical studies. Sections (5 μm thick) were made, deparaffinized, rehydrated in PBS, placed in 10 mmol/l citrate buffer (pH 6.0), and heated in a 750 W microwave four times for 6 min. The endogenous peroxidase activity was blocked by incubation for 10 min in 0.3% H_2O_2. After washing three times with PBS, the sections were then preincubated with 2% bovine serum albumin in PBS for 40 min at room temperature and then incubated with primary antibodies, anti-β-catenin (1:1000; BD Biosciences PharMingen, San Diego, CA, USA), anti-Cox-2 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Subsequently, the sections were incubated with biotinylated secondary antibodies against the primary antibodies (1:250; DAKO Corp., Carpinteria, CA, USA) for 30 min followed by incubation with avidin-coupled peroxidase (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. The sections were developed with 3,3′-diaminobenzidine (DAB) using DAKO Liquid DAB Substrate-Chromogen System (DAKO) and then were counterstained with hematoxylin, dehydrated, and coverslipped.

2.5. *Protein extraction and a Western blot analysis*

Total protein was extracted from both the normal colon tissues and the colon tumor tissues, which were excised from *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice at autopsy (Fig. 1a), and equivalent amounts of protein (15 μg/lane) were subjected to a Western blot analysis, as described previously [24,25]. The primary antibodies for β-catenin, GAPDH were purchased from BD Biosciences PharMingen (San Diego, CA, USA) and Cell Signaling Technology, Inc. (Danvers, MA, USA), respectively. An antibody against GAPDH served as a loading control.

2.6. *Crypt isolation*

The excised total colon was washed by PBS several times and cut opened in the longitudinal direction. The total colon was divided into three sections and the distal section was used for crypt isolation. The distal tissue was washed by 1× Hank's Balanced Salt Solution (HBSS; Sigma–Aldrich, St Louis, MO, USA) two times and followed by incubation with 1× HBSS containing 30 mM EDTA at 37 °C for 15 min. After this step, the tissue was dispersed by vortex in the 1× HBSS solution and separated into epithelial crypts and stromal tissues.

2.7. *Quantitative real-time reversed transcription-polymerase chain reaction*

Total RNA was extracted from the isolated epithelial crypts and stromal tissues of wild-type C57B6/J and Tnfα −/− mice at the indicated time intervals (Fig. 1b) by using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. cDNA was synthesized from 1.0 μg of total RNA by using SuperScript III First-Strand Synthesis System (Invitrogen Life Technologies, Carlsbad, CA, USA). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) amplification was performed in a final volume of 20 μl containing 10 μl of 2× SYBR green master mix (Takara, Kyoto, Japan), 1.0 μl of primers (10 μmol/l), 3.0 μl of distilled water and 5.0 μl of cDNA by using a LightCycler 1.0. (Roche Diagnostics, IN, USA) according to the protocols described previously [26]. The reaction conditions included activation at 95 °C for 10 min, denaturation at 95 °C for 10 s, annealing 60 °C for 10 s and extension 72 °C for 6 s. All PCR amplifications were done for 45 cycles. The expression level of each gene was normalized to the β-actin expression level using the standard curve method. The primer sequences used in qRT-PCR analyses were obtained from the PrimerBank (http://pga.mgh.harvard.edu/primerbank/): for *β-actin*, sense 5′-CATCCGTAAAGACCTCTATGCCAAC-3′ and antisense 5′-ATGGAGCCACCGATCCACA-3′; for *Tnfα*, sense 5′-CCCTCACACTCAGATCATCTTCT-3′ and antisense 5′-GCTACGACGTGGGCTACAG-3′; for *Tnfβ*, sense 5′-CCACCTCTTGAGGGTGCTTG-3′ and antisense 5′-CATGTCGGAGAAAGGCACGAT-3′; for *Ccl2*, sense 5′-TTAAAAACCTGGATCGGAACCAA-3′ and antisense 5′-GCATTAGCTTCAGATTTACGGGT-3′; for *Cxcl1*, sense 5′-CTGGGATTCACCTCAAGAACATC-3′ and antisense 5′-CAGGGTCAAGGCAAGCCTC-3′; for *Cox*-2, sense 5′-TGAGCAACTATTCCAAACCAGC-3′ and antisense 5′-GCACGTAGTCTTCGATCACTATC-3′; for *Il1β*, sense 5′-GCAACTGTTCCTGAACTCAACT-3′ and antisense 5′-ATCTTTTGGGGTCCGTCAACT-3′; and for *Il6*, sense 5′-TAGTCCTTCCTACCCCAATTTCC-3′ and antisense 5′-TTGGTCCTTAGCCACTCCTTC-3′.

2.8. *Statistical analysis*

The statistical analysis was performed using the GraphPad Prism 4 software program (Graphpad Software, Inc., San Diego, CA, USA). The mean ± SD was calculated for all parameters determined. Statistical significance was evaluated using either Student's *t*-test or Welch's *t*-test for paired samples. *p* values <0.05 were considered to be statistically significant.

3. Results

3.1. *Genetic ablation of Tnfα did not suppress both the colonic inflammation and the expression of pro-inflammatory factors in the colon of mice exposed to DSS*

A previous study revealed that *Tnfr1* deficient mice are resistant to the DSS-induced colitis. In the present study, two cohorts of *Apc* Min/+ mice with different *Tnfα* status (*Apc* Min/+; *Tnfα* +/+, *Apc* Min/+; *Tnfα* −/− mice) were exposed to 2% DSS in drinking water for 1 week and sacrificed at the point of week 5 (Fig. 1a). In contrast to the *Tnfr1* deficient mice, mice lacking *Tnfα*, as well as the control mice, exhibited profound body weight loss and bloody diarrhea during the course of DSS treatment. In addition, there were no significant differences in either the physical findings or mortality between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice. There were no apparent differences in either microscopic appearance of the inflamed colon (data not shown) or the histological inflammation score (1.17 ± 0.83 and 1.25 ± 0.97, respectively, *p*: 0.82) between these two cohorts.

We also examined the effect of *Tnfα* ablation on intestinal inflammation using the trinitrobenzene sulfonic acid (TNBS)-induced model, a different model of intestinal inflammation. However, we could not detect any differences in the histological inflammation score between *Tnfα* +/+ and *Tnfα* −/− mice (2.75 ± 1.50 and 2.50 ± 1.22, respectively, *p*: 0.78).

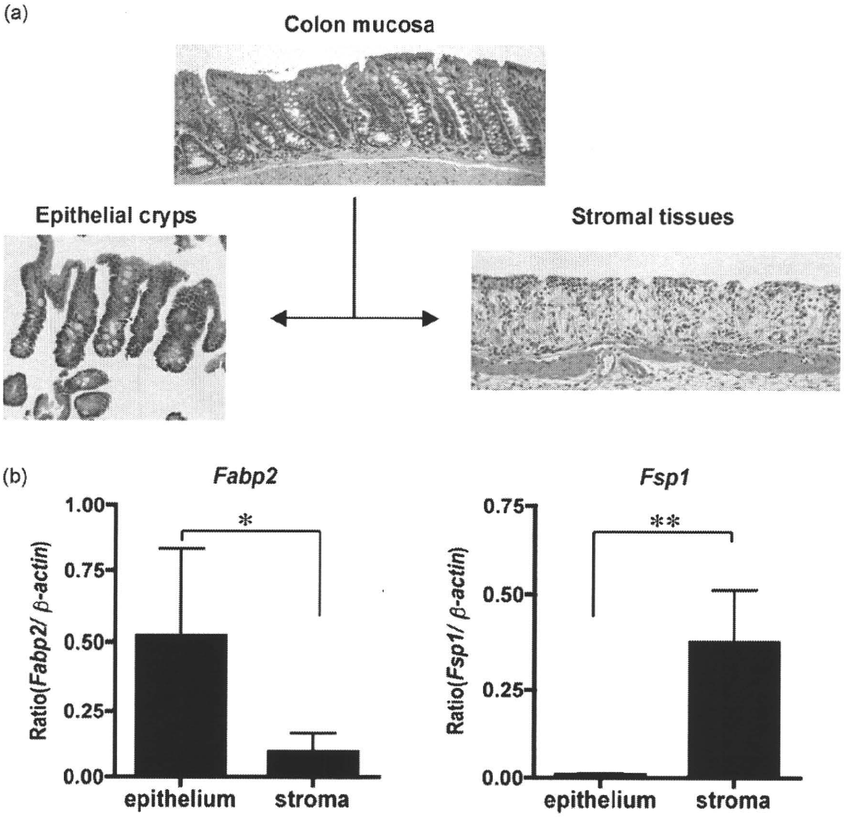

Fig. 2. Crypt isolation and the expression of epithelial or stromal marker gene. (a) Distal colon tissues were separated into epithelial crypts and stromal tissues by crypt isolation as described in Section 2. (b) Quantitative RT-PCR was performed on total RNAs extracted from both epithelial cryps and stromal tissues. The levels of FABP2 and FSP-1 mRNA were normalized to β-actin mRNA levels. Representative results from three independent experiments are shown here. Data represent the mean ± SD. Statistical significance of differences was evaluated by Student's *t*-test with Welch's correction. $^{*}p < 0.05$, $^{**}p < 0.01$.

Both wild-type and *Tnfα* deficient mice were treated with 2% DSS for 5 days and then were sacrificed at day 0 (control), day 10 (the acute phase of colonic inflammation) and day 30 (the chronic phase of colon inflammation; Fig. 1b) in order to compare the induction of pro-inflammatory factors in the presence or absence of *Tnfα*. The crypts were isolated to separate the colons into the epithelial crypts and stromal tissues (Fig. 2a). After crypt isolation, RNA was extracted from these tissue specimens and real-time PCR was used to compare the expression of pro-inflammatory factors between wild-type and *Tnfα* deficient mice. The epithelial marker, *fatty-acid-binding-protein*-2 (*Fabp*2) [27–29], was predominantly expressed by RNAs from colonic crypts, whereas the mesenchymal marker, *fibroblast-specific-protein*-1 (*Fsp*1), was predominantly expressed at those from stromal tissues (Fig. 2b). These findings indicate that epithelial cells were therefore successfully separated from stromal cells.

The expression of pro-inflammatory factors (*Tnfα*, *Tnfβ*, *Ccl*2, *Cxcl*1, *Il*1*β*, *Il*6, *Cox*-2) were up-regulated after the DSS exposure in wild-type mice and such altered expression was maintained at a high level until day 30 (Fig. 3a). As expected, during the course of DSS treatment (Fig. 1b), the expression of *Tnfα* was not detectable in either the epithelial or stromal tissues of *Tnfα* deficient mice (Fig. 3b). However, in spite of the lack of *Tnfα* expression, there were no significant differences in the expression of *Tnfβ*, *Ccl*2, *Cxcl*1, *Il*1*β*, *Il*6 and *Cox*-2 in either the epithelial or stromal tissues between the wild-type and *Tnfα* deficient mice (Fig. 3c). These results indicate that the genetic ablation of *Tnfα* did not influence either the severity of colitis or the expression of DSS-induced pro-inflammatory factors.

3.2. Genetic ablation of *Tnfα* did not suppress the infiltration of Cox-2-expressing inflammatory cells and the accumulation of β-catenin protein in the inflamed colorectal tumor tissues

The coordinated activation of the Apc/β-catenin pathway and the Cox-2 signaling pathway plays an important role in colon tumor formation and progression [30]. A previous study revealed a lack of *Tnfr*1 to lead to the decreased expression of Cox-2 in the stromal tissues and the decreased expression of nuclear β-catenin in the colonic tumor cells [11]. Therefore, the infiltration of Cox-2-expressing inflammatory cells and the accumulation of nuclear β-catenin was examined by immunostaining. In contrast to previous data, no significant differences were observed in the infiltration index of Cox-2-positive inflammatory cells between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice (42.62 ± 6.65 and 45.73 ± 2.90 cells per field at 400× magnification, respectively, *p*: 0.32) (Fig. 4a). The accumulation of nuclear β-catenin protein in colon tumor cells was not different between the two cohorts (Fig. 4b). In addition, Western blotting revealed the expression of β-catenin protein in colonic tumors to not change between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice (Fig. 4c).

3.3. The genetic ablation of *Tnfα* did not suppress the inflammation-related colon tumorigenesis

*Tnfr*1 deficient mice markedly attenuate tumor formation induced by azoxymethane (AOM) and DSS in comparison to wild-type mice [11]. In the present study, as in a previous report [20], colon tumors were detectable only in *Apc* Min/+ mice exposed to

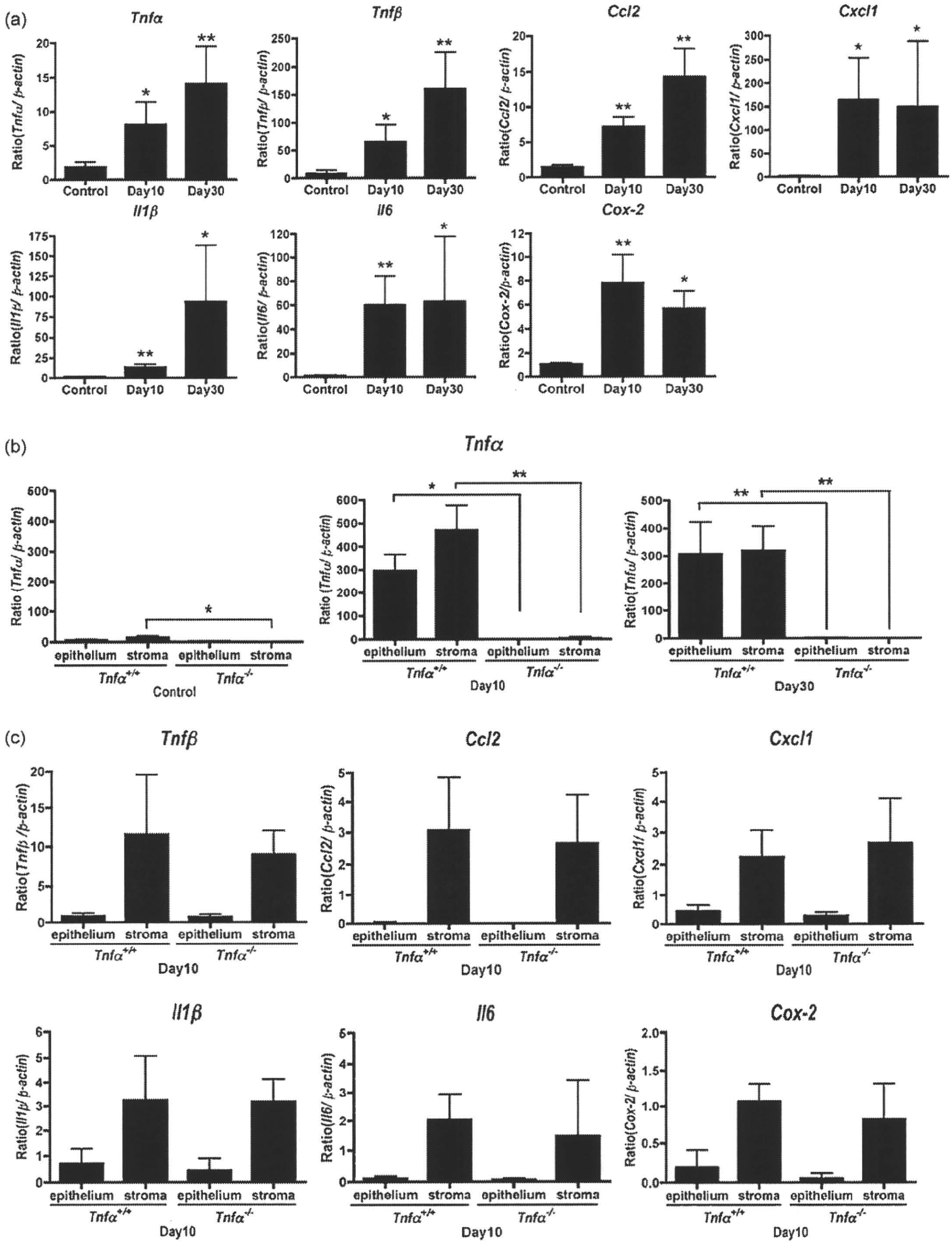

Fig. 3. Pro-inflammatory factor gene expression in the colon. Quantitative RT-PCR was performed on total RNAs extracted from stromal tissues of wild-type mice (a), epithelial and stromal tissues of both wild-type and Tnfα$^{-/-}$ mice (b and c) at the indicated time intervals as indicated. The levels of each pro-inflammatory factor were normalized to β-actin mRNA levels. Representative results from three independent experiments are shown here. Data represent the mean ± SD. Statistical significance of differences was evaluated by Student's *t*-test with Welch's correction (a–c). $^*p < 0.05$, $^{**}p < 0.01$.

DSS, whereas no tumors were found in *Apc* +/+ mice with the DSS exposure. The incidence and multiplicity of colonic tumors were 100% and 16.15 ± 5.84/mouse in *Apc* Min/+; *Tnfα* +/+ mice and 100% and 14.27 ± 7.71/mouse in *Apc* Min/+; *Tnfα* −/− mice, respectively. The tumor volumes were 3.90 ± 2.55 mm^3 and 3.81 ± 2.49 mm^3 in *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice, respectively (Table 1). Importantly, no significant differences were observed in either the tumor incidence and multiplicity or the tumor volumes between *Apc* Min/+; *Tnfα*+/+ and *Apc* Min/+; *Tnfα*−/− mice. Microscopic examinations also failed to detect any histological differences in colon tumors between *Apc* Min/+; *Tnfα* +/+ and *Apc* Min/+; *Tnfα* −/− mice (data not shown). In addition, the incidence and multiplicity of small intestinal tumors did not alter regardless of the *Tnfα* status (100% and 47.0 ± 2.16/mouse in *Apc* Min/+; *Tnfα* +/+ mice and 100% and 48.8 ± 3.59/mouse in *Apc* Min/+; *Tnfα* −/− mice, respectively).

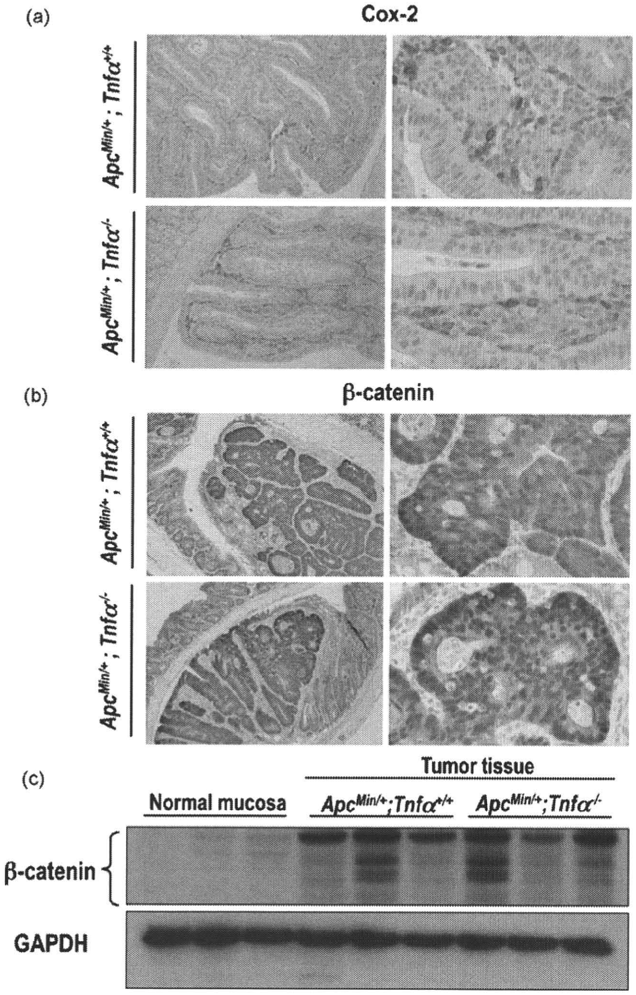

Fig. 4. Cox-2-expressing cells and β-catenin nuclear accumulation in the colon tumor tissues. Colons were immunostained with anti-Cox-2 (a) or anti-β-catenin antibody (b) and representative results from five independent animals are shown here. Original magnification, 100× (left panels), 400× (right panels), respectively. (c) Western blot analysis with anti-β-catenin antibody was performed on cell lysates from both normal colon mucosa and colon tumor tissues. Representative results from three independent experiments are shown here.

4. Discussion

Tnfα is a hormone with a broad spectrum of biological activities, produced mainly by activated macrophages and a variety of other cell types, including activated T cells, mast cells, neutrophils, and astrocytes [31]. Once the protein is efficiently exported from the producing cell, it enters the circulation, where it has a very limited half-life and binds to either high-affinity 55-kDa TNF-receptor-1 (Tnfr1) or low-affinity 75-kDa TNF-receptor-2 (Tnfr2) [32,33]. Popivanova et al. revealed that mice lacking *Tnfr*1 treated with AOM and DSS showed reduced mucosal damage, reduced infiltration of macrophages and neutrophils, and attenuated subsequent tumor formation [11]. These findings indicate Tnfα/Tnfr1 signaling as a crucial mediator of the initiation and promotion of colitis-associated colon carcinogenesis, and suggest that targeting Tnfα may be a useful strategy for prevention and/or treatment of colon cancer in the individuals with IBD.

Table 1
Incidence, multiplicity and tumor volume of large intestinal tumors at week 5.

Genotype	Incidence	Multiplicity[a]	Tumor volume[b]
$Apc^{Min/+}$; $Tnf\alpha^{+/+}$	26/26, 100%	16.15 ± 5.84	3.90 ± 2.25
$Apc^{Min/+}$; $Tnf\alpha^{-/-}$	25/25, 100%	14.27 ± 7.71	3.81 ± 2.49

Statistical significance of differences was evaluated by Student's *t*-test.

[a] Number of tumors/mouse, the mean ± SD.

[b] Tumor volume was calculated as length × width × width × 0.526, the mean ± SD.

In contrast [11], the current study revealed that, despite a deficiency in the *Tnfα* expression, both chemically induced colonic inflammation and the tumor formation in *Tnfα* deficient mice were not attenuated in comparison to those of *Tnfα* +/+ control mice. In addition, the expression of pro-inflammatory factors in the colon mucosa exposed to DSS was not altered in comparison to wild-type control mice. Moreover, no significant differences were observed in either the infiltration of Cox-2-positive inflammatory cells or the nuclear β-catenin accumulation of tumor cells between *Apc* Min/+:*Tnfα* +/+ and *Apc* Min/+:*Tnfα* −/− mice, both of which have been shown to be significantly suppressed in *Tnfr*1 deficient mice [11].

Although Tnfα is the ligand for the Tnfr1, it is noteworthy that Tnfr1 also binds with tumor necrosis factor-β (Tnfβ) or lymphotoxin (LT) [34]. Tnfβ shares about 30% structural homology with Tnfα [35,36], and Tnfα and Tnfβ are functionally indistinguishable with respect to receptor binding and activation of Nf-kB in HL60 cells [37]. Although DSS-induced *Tnfβ* expression was almost equal in both wild-type and *Tnfα* deficient mice, it might be possible that Tnfβ is involved in the induction of colonic inflammation and inflammation-related colon tumorigenesis induced by DSS. This finding is consistent with the previous findings that activation of Nf-kB is associated with DSS-induced inflammation-related colon tumorigenesis [13,38] and that the maximum activation of Nf-kB with Tnfα and/or Tnfβ requires only a small fraction of the total number of Tnf-receptors to be occupied [39].

Previous studies demonstrated that several inflammation-related factors including *Il*1β, Stat3 and iNOS are involved in DSS-induced inflammation-related colon tumorigenesis [38,40,41]. In addition, the possible interaction between iNOS, TnFα and *Il*1β is suggested to play a role in the tumor promotion of inflammation-related colon carcinogenesis [42]. Given such intricate inflammatory responses, it is also possible that the Tnfα-independent signal may promote the inflammation-related mouse colon tumorigenesis in the present study.

There is increasing evidence that the anti-Tnfα monoclonal antibody, infliximab, is an effective therapy for IBD, including Crohn's disease and ulcerative colitis [43–49]. Infliximab is a chimeric monoclonal antibody that binds not only the soluble subunit of Tnfα but also the membrane-bound precursor of Tnfα [50,51]. Infliximab inhibits a broad range of biological activities of Tnfα by blocking the interaction of Tnfα with its receptors. Given the fact that the genetic deletion for *Tnfr*1 significantly suppressed inflammation-related colon tumorigenesis, it was expected that the blockage for Tnfα with the use of infliximab could be a useful strategy for both the chemoprevention and therapy for tumorigenesis. Indeed, previous studies demonstrated that antibodies against Tnfα strongly suppress the development of inflammation-related CRC [11,15]. In contrast, the current study revealed that the genetic ablation of Tnfα alone did not either reduce colon inflammation or attenuate tumor formation. Our results may therefore suggest that an indirect action of anti-Tnfα antibodies exerts a tumor suppressive effect in inflammation-related CRC. A previous study demonstrated that anti-Tnfα monoclonal antibody binds to the transmembrane form of Tnfα, thus resulting in the efficient killing of the Tnfα-expressing cells by both antibody-dependent cellular toxicity and complement-dependent cytotoxicity effector mechanisms [51,52]. The mode of action of anti-Tnfα monoclonal antibody for the treatment of IBD and IBD-related tumorigenesis might be attributed principally to the lysis of the inflammatory cells rather than blocking the interaction of Tnfα with its receptors.

In conclusion, the current study revealed that the genetic ablation of *Tnfα* results in no detectable effect on either the suppression of DSS-induced colonic inflammation or the attenuation of inflammation-related mouse colon tumorigenesis. These observations suggest that intricate inflammatory responses are involved in the inflammation-related mouse colon tumorigenesis.

Conflicts of interest

No conflicts of interest.

Acknowledgments

We would like to thank Masanao Saio and Yusuke Kitou for helpful discussions. We would also thank Kyoko Takahashi, Ayako Suga, and Yoshitaka Kinjyo for their valuable technical assistance and animal care. This study was supported by grants from Japan Science and Technology (JST), grants from PRESTO, grants from the Ministry of Health, Labour and Welfare of Japan, and grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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INTERNATIONAL JOURNAL OF ONCOLOGY 36: 585-592, 2010

Rho-kinase regulates negatively the epidermal growth factor-stimulated colon cancer cell proliferation

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Received October 16, 2009; Accepted December 3, 2009

DOI: 10.3892/ijo_00000533

Abstract. It has been reported that Rho and Rho-kinase are involved in actin cytoskeleton organization and associated with carcinogenesis and progression of human cancers. However, the mechanism how the Rho/Rho-kinase pathway is involved in cell cycle progression has not been precisely characterized. In this study, we investigated the role of Rho-kinase in epidermal growth factor (EGF) signaling in SW480 colon cancer cells. We found that Y27632, a Rho-kinase inhibitor, dose-dependently induced cell proliferation in these cells. The blockade of EGF stimulation utilizing anti-EGF receptor neutralizing antibodies significantly suppressed cell growth, suggesting that EGF stimulation plays an important role in cell proliferation in SW480 cells. We also found that EGF induced Rho-kinase activation. Interestingly, EGF-induced phosphorylation of both Akt and glycogen synthase kinase-3ß (GSK-3ß), but not p44/p42 mitogen-activated protein (MAP) kinase, were dose-dependently enhanced when the cells were pretreated with Y27632 or fasudil, another Rho-kinase inhibitor. Moreover, whereas EGF increased the phosphorylation of retinoblastoma tumor suppressor protein as well as cyclin D1 protein expression level, pretreatment with Y27632 accelerated them. Taken together, our results suggest that Rho-kinase regulates negatively EGF-induced cell proliferation upstream of Akt/GSK-3ß in colon cancer cells.

Introduction

GTPases of the Ras and Rho superfamily act as molecular switches to control a wide range of essential biological pathways in all eukaryotic cells (1). In their active, GTP-bound state, Ras and Rho GTPases interact with target proteins to promote a cellular response (1). Among the super-family, Rho regulates the formation of contractile actin-myosin filaments, which form stress fibers, and maintains focal adhesions at the rear of the cell (2). Increasing evidence implicates Rho GTPases as major players in the regulation of a variety of cellular processes in addition to contraction (3). Rho GTPases reportedly contribute to the regulation of cell cycle progression, cell motility and invasive phenotypes (3). Although overexpression of Rho has been linked to progression of human cancers (4), the roles of the Rho GTPases and their downstream underlying cancer progression and invasion remain controversial.

Rho-kinase is one of the various targets to Rho (5). Accumulating evidence indicates that the Rho/Rho-kinase pathway plays an important role in various cellular functions such as vascular smooth muscle cell contraction, cell migration and cell proliferation (3). Action of the Rho/Rho-kinase pathway takes part in cancer progression by regulating actin cytoskeleton reorganization. Since a specific Rho-kinase inhibitor was found to suppress tumor growth and metastasis (6), it is generally recognized that the Rho/Rho-kinase pathway may become a molecular target for prevention of cancer invasion and metastasis.

On the other hand, the ability of cancer cells for survival is dependent largely on the epidermal growth factor (EGF) (7). Through its binding to cell surface EGF receptor (EGFR), EGF activates an extensive network of signal transduction pathways that include activation of the Ras/p44/p42 mitogen-activated protein (MAP) kinase and phosphatidyl inositol-3-kinase (PI3K)/Akt pathways (7). Glycogen synthase kinase (GSK)-3 is a critical downstream element of the PI3K/Akt pathway, and its activity can be inhibited by Akt-mediated phosphorylation of GSK-3α at Ser21 and GSK-3ß at Ser9 (8,9). Unlike most protein kinases, GSK-3ß is typically active in unstimulated cells (10). Thus, GSK-3ß phosphorylates Thr286 of cyclin D1 (11) and phosphorylated cyclin D1 is exported from the nucleus and degraded by the 26S proteasome system after ubiquitination (12). It has also been demonstrated that activation of PI3K and Akt-mediated phosphorylation of GSK-3ß negatively regulate its activity, thus promoting increased cyclin D1 protein levels (11). Furthermore, cyclin D1 complex with cyclin-dependent kinase (CDK) 4 and CDK6 stimulate their kinase activities, which in turn cause the phosphorylation and inactivation of the retinoblastoma tumor-suppressor protein (Rb) (11), directing toward cell proliferation. However, the

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Key words: Rho-kinase, EGF, cell growth, colon cancer

relationship between Rho-kinase and EGF signaling in cancer cells has not been precisely clarified.

In the present study, we investigated the role of Rho-kinase in EGF-induced colon cancer cell progression. Interestingly, we found for the first time that Rho-kinase plays a negative role in EGF-stimulated proliferation of colon cancer cells.

Materials and methods

Materials. Y27632 was obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). EGF and hydroxyfasudil (fasudil) were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-phospho-specific myosin phosphatase targeting subunit-1 (MYPT-1) (Thr850) antibodies were purchased from Upstate (Lake Placid, NY). Anti-MYPT-1 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phospho-specific Akt, Akt, phospho-specific GSK-3ß, GSK-3ß, cyclin D1, phospho-specific Rb and phospho-specific myosin regulatory light chain (MLC) were purchased from Cell Signaling Inc. (Beverly, MA). Anti-EGFR-neutralizing antibodies were purchased from Millipore (Temecula, CA). ECL Western blot detection system was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. Y27632 and fasudil were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of DMSO was 0.1%, which did not affect the assay for Western blot analysis.

Cell culture and chemicals. Unless indicated otherwise SW480 human colon cancer cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, San Diego, CA), containing 10% fetal calf serum (FCS). Before the experiments, they were incubated in serum-free medium for an additional 24 h as described previously (13).

Cell proliferation assay. Cell proliferation assay was performed using cell proliferation ELISA (5-bromo-deoxy-uridine: BrdU) and 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) cell proliferation kit I (Roche Diagnostics Co., Indianapolis, IN), according to the instructions of the manufacturer, respectively. In brief, SW480 cells were plated onto 96-well plates (3×10^3 cells/well) and 24 h later, the cells were treated with the indicated doses (0-10 μM) of Y27632 for 48 h in DMEM containing 10% FCS. The medium and agents were not changed during this time period. In Fig. 1C, the attached cells were treated with 0.5 μg/ml of anti-EGFR-neutralizing antibodies or normal mouse-IgG for 5 days under medium containing 3% FCS and then the remaining cells were counted by MTT cell proliferation kit I. All assays were done in triplicate.

Western blot analysis. Western blot analysis was performed as described previously (13). The cultured cells were pretreated with various doses of Y27632 or fasudil for 60 min, and then stimulated by 30 ng/ml of EGF for the indicated periods. Protein extracts were examined by Western blot analysis. The protein (12 μg) was fractionated and transferred onto an Immuno-Blot PVDF Membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% fat-free dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) for 30 min before incubation with the indicated primary antibodies. Peroxidase-labeled antibodies raised in goat against rabbit IgG were used as second antibodies. Peroxidase activity on the membrane was visualized on X-ray film by means of the ECL Western blot detection system.

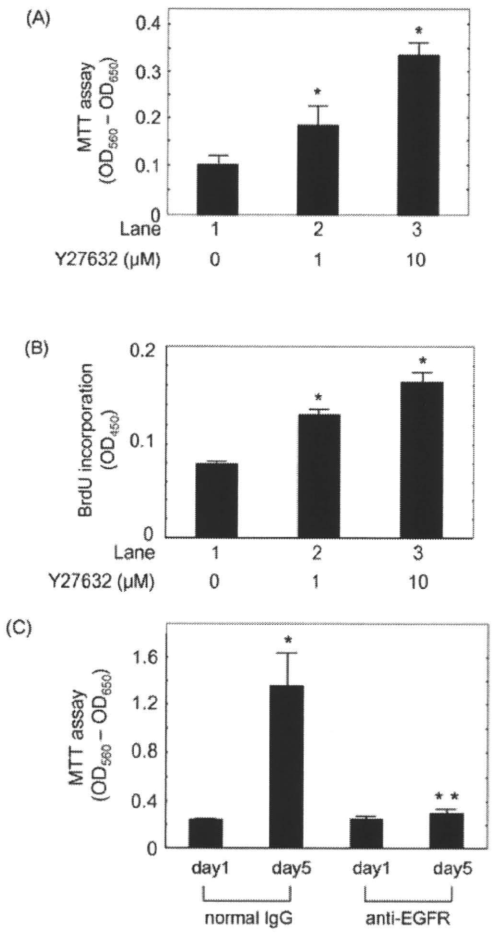

Figure 1. Effects of Y27632 on anti-EGFR-neutralizing antibodies in SW480 colon cancer cell proliferation. (A) Cell viability assay was performed using the MTT cell proliferation kit I. Results are expressed as the absorbance (OD 560 nm-OD 750 nm). (B) The measurement of BrdU incorporation during DNA synthesis was also performed using cell proliferation ELISA (BrdU). Results are expressed as the absorbance (OD 450 nm). Bars designate SD of triplicate assays. (C) The attached cells were treated with 0.5 μg/ml of anti-EGFR-neutralizing antibodies or normal mouse-IgG for 5 days under medium containing 3% FCS and then the remaining cells were counted by MTT cell proliferation kit I. All assays were done in triplicate.

Immunofluorescence microscopy studies. Immunofluorescence microscopy studies were performed as described previously (14). The cells grown on coverslip-bottom dishes were treated with or without Y27632 (10 μM) for 1 h at 37°C, followed by exposure to 30 ng/ml of EGF for 15 min at 37°C. They were then fixed with 4% paraformaldehyde for 10 min on ice and then exposed to 0.1% Triton X-100 for 10 min to

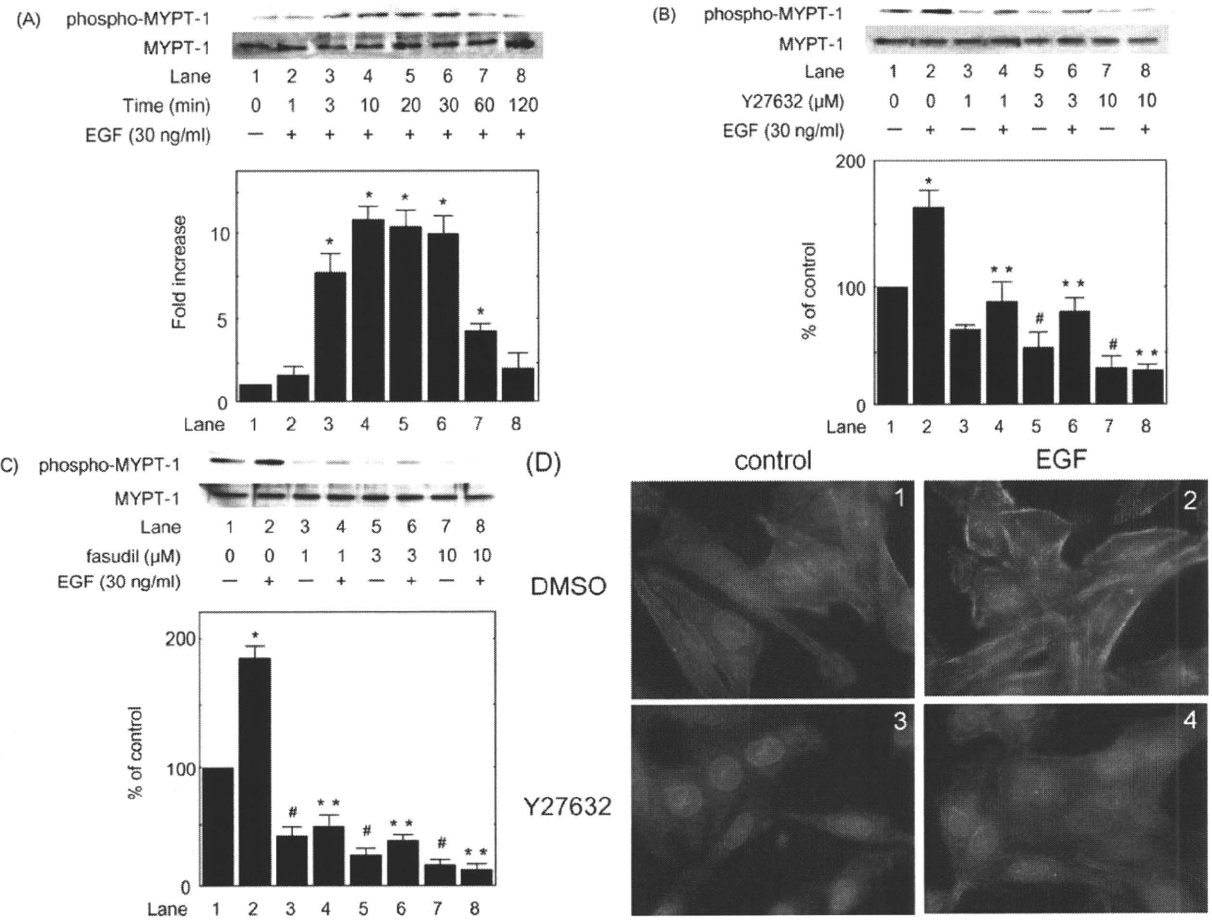

Figure 2. Effects of Rho-kinase inhibitors on EGF-induced phosphorylation of MYPT-1 in SW480 colon cancer cells. (A) SW480 cells were stimulated with 30 ng/ml EGF for the indicated periods. The lower bar graph shows the quantification data for the relative levels of phospho-MYPT-1, after normalization with respect to total MYPT-1, as determined by densitometry. The asterisks indicate significant increase (*p<0.05, compared with the control). (B and C) SW480 cells were pretreated with various doses of Y27632 (B) or fasudil (C) for 60 min, and then stimulated with 30 ng/ml EGF or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific MYPT-1 or MYPT-1. The lower bar graph shows the quantification data for the relative levels of phospho-MYPT-1, after normalization with respect to total MYPT-1, as determined by densitometry. (*) indicates significant increase (p<0.05), as compared with the control (lane 1), respectively. (**) indicates significant decrease (p<0.05), as compared with the control (lane 2), respectively. ($^{\#}$) indicates significant decrease (p<0.05), as compared with the control (lane 1), respectively. (D) SW480 cells grown on coverslip-bottom dishes were treated with or without Y27632 (10 μM) for 60 min at 37˚C, followed by exposure to 30 ng/ml of EGF for 20 min at 37˚C. After fixation, they were then exposed to anti-phospho MLC antibodies, followed by exposure to Alexa Fluor 488-conjugated goat anti-rabbit IgG antibodies and DAPI for 1 h. The cells were then examined by fluorescence microscopy.

permeabilize the cell membrane. They were then exposed to anti-phospho MLC antibodies, followed by exposure to Alexa Fluor 488® conjugated goat anti-rabbit IgG antibodies and 4',6-diamidino-2-phenylindole (DAPI) (Wako, Tokyo, Japan) for 1 h. The cells were then examined by fluorescence microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan) according to the manufacturer's protocol.

Densitometric analysis. The densitometric analysis was performed using scanner and image analysis software (image J ver. 1.32). The background subtracted signal intensity of each protein signal was normalized by the respective control signal. The data were obtained from at least three independent experiments.

Results

Effect of Y27632 on cell proliferation in SW480 colon cancer cells. In order to clarify whether Rho-kinase is involved in colon cancer cell proliferation, we first performed cell proliferation assay using MTT and BrdU in SW480 cells grown in medium containing 10% FCS. We used Y27632 as a specific Rho-kinase inhibitor (15) and found in MTT cell proliferation assay that the cell growth was dose-dependently enhanced when the cells were treated with Y27632 (Fig. 1A). As well, BrdU incorporation was also increased in Y27632-treated SW480 cells (Fig. 1B). Therefore, it is likely that Rho-kinase plays a suppressive role in SW480 cell proliferation.

Effect of anti-EGFR-neutralizing antibodies on cell proliferation in SW480 colon cancer cells. We next examined the

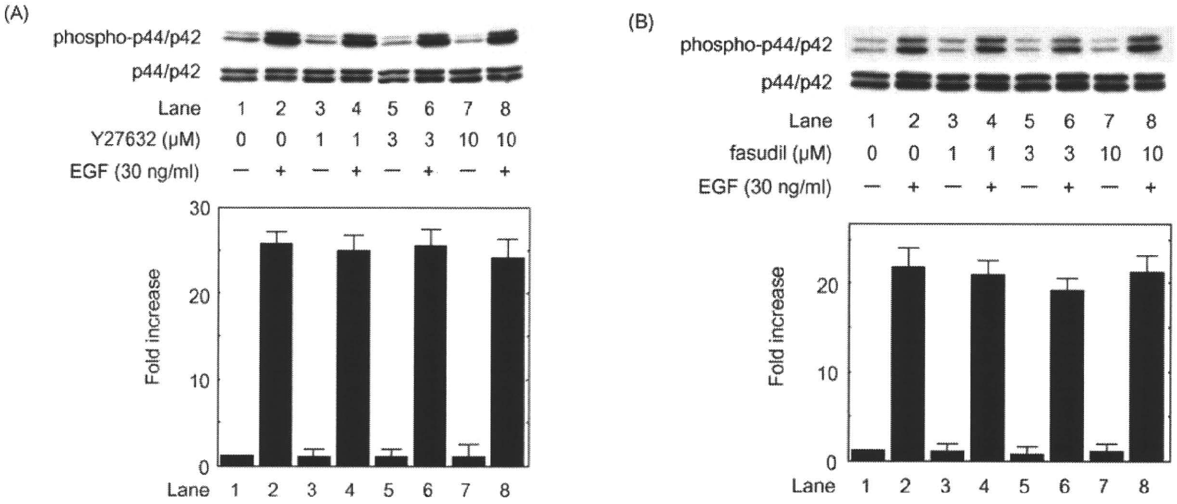

Figure 3. Effects of Rho-kinase inhibitors on EGF-induced phosphorylation of p44/p42 MAP kinase in SW480 colon cancer cells. The cells were pretreated with the indicated doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated with 30 ng/ml EGF or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The lower bar graph shows the quantification data for the relative levels of phospho-p44/p42 MAP kinase, after normalization with respect to total p44/p42 MAP kinase, as determined by densitometry.

effect of the blockade of EGF stimulation utilizing anti-EGFR-neutralizing antibodies on cell proliferation in SW480 cells grown in medium containing 3% FCS. As expected, when the cells were treated with anti-EGFR-neutralizing antibodies for 5 days, cell growth was significantly suppressed, as compared with the cells treated with normal mouse-IgG (Fig. 1C). We also obtained similar results when the cells were cultured in medium containing 10% FCS (data not shown). Since medium containing 3% FCS is recognized to contain various types of growth factors including EGF, it is most likely that EGF stimulation plays an important role in cell proliferation in SW480 cells. Moreover, these results led us to further investigate the role of Rho-kinase in EGF-treated colon cancer cells.

Effects of EGF on the phosphorylation of MYPT-1 in SW480 colon cancer cells. MYPT-1, which is a component of myosin phosphatase, is well known as a downstream substrate of Rho-kinase (5). In order to clarify whether EGF activates Rho-kinase in SW480 colon cancer cells, we examined the effect of EGF on the phosphorylation of MYPT-1. EGF markedly induced the phosphorylation of MYPT-1 in a time-dependent manner (Fig. 2A). The effect of EGF on the phosphorylation of MYPT-1 reached its maximum at 10 min, sustained up to 30 min, and decreased thereafter. We next confirmed whether Y27632 suppresses the EGF-induced phosphorylation levels of MYPT-1. Y27632 (1 μM) partially attenuated (Fig. 2B, lane 4 compared to lane 2) and 10 μM of Y27632 almost completely suppressed the EGF-induced phosphorylation levels of MYPT-1 (Fig. 2B, lane 8). Interestingly, we observed that the basal level of MYPT-1 phosphorylation in unstimulated cells (Fig. 2B, lane 1) was also suppressed by pretreatment with Y27632 (Fig. 2B, lanes 5 and 7 compared to lane 1). Similarly, fasudil, another inhibitor of Rho-kinase (15), also attenuated the EGF-induced phosphorylation levels of MYPT-1 (Fig. 2C).

Effects of EGF on the phosphorylation of MLC in SW480 colon cancer cells. Rho is implicated in the cytoskeletal responses to extracellular signals lysophosphatidic acid and certain growth factors, which form stress fibers and cause focal adhesion (16,17). It has been shown that Rho-kinase phosphorylates the MLC of smooth muscle (18), indicating that phosphorylation of MLC reflects Rho-kinase activity. To further confirm whether EGF activates Rho-kinase in SW480 cells, we examined the effect of EGF on the phosphorylation of MLC in an immunofluorescence study. When the cells were stimulated by 30 ng/ml of EGF for 20 min, phosphorylated MLC levels were clearly strengthened in cells (Fig. 2D, panel 2 compared to panel 1). The basal level of MLC phosphorylation was suppressed by 10 μM of Y27632 (Fig. 2D, panel 3). Moreover, pretreatment with Y27632 markedly reduced EGF-induced MLC phosphorylation (Fig. 2D, panel 4). Taken together with the results shown in Fig. 2B and C, we strongly suggest that EGF induces activation of Rho-kinase in SW480 colon cancer cells.

Effects of Y27632 or fasudil on the EGF-induced phosphorylation of p44/p42 MAP kinase in SW480 colon cancer cells. It is generally recognized that p44/p42 MAP kinase is a central element used by mammalian cells to transduce the proliferative message of a variety of growth factors (19). We therefore examined the effect of EGF on the phosphorylation of p44/p42 MAP kinase in SW480 cells. We found that EGF markedly induced the phosphorylation of p44/p42 MAP kinase in a time-dependent manner (data not shown) as previously shown in colon cancer cells (20). We next examined the effect of Y27632 or fasudil on the EGF-induced phosphorylation of p44/p42 MAP kinase. However, neither Y27632 nor fasudil affected the EGF-induced phosphorylation of p44/p42 MAP kinase in the range between 1 and 10 μM (Fig. 3A and B, upper panel, respectively). Therefore, it seems unlikely that Rho-kinase is

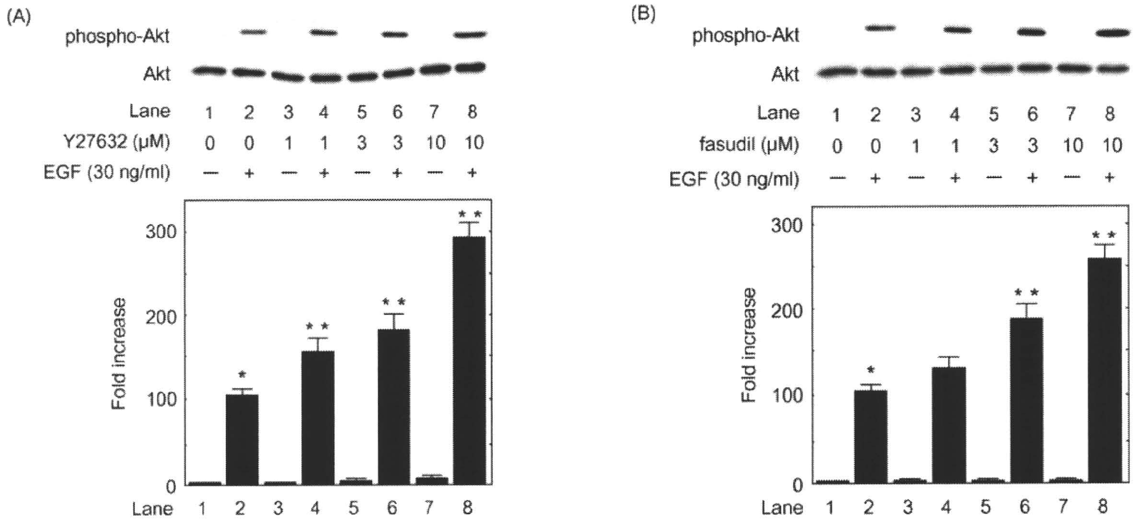

Figure 4. Effects of Rho-kinase inhibitors on EGF-induced phosphorylation of Akt in SW480 colon cancer cells. The cells were pretreated with the indicated doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated with 30 ng/ml EGF or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific Akt or Akt. The lower bar graph shows the quantification data for the relative levels of phospho-Akt, after normalization with respect to total Akt, as determined by densitometry. (*) indicates significant increase ($p<0.05$), as compared with the control (lane 1), respectively. (**) indicates significant increase ($p<0.05$), as compared with the control (lane 2), respectively.

involved in EGF-induced phosphorylation of p44/p42 MAP kinase.

Effects of Y27632 or fasudil on the EGF-induced phosphorylation of Akt in SW480 colon cancer cells. It is well-known that Akt regulates multiple biological processes including survival, proliferation and cell growth (21). In general, Akt promotes cell survival by inhibiting apoptosis by phosphorylating and inactivating several targets, including Bad (22), forkhead transcription factors (23) and c-Raf (24). We found that EGF markedly induced the phosphorylation of Akt in a time-dependent manner (data not shown) as previously shown in colon cancer cells (25). We next examined the effect of Y27632 or fasudil on the EGF-induced phosphorylation of Akt. Surprisingly, EGF-induced Akt phosphorylation was markedly enhanced when the cells were pretreated with Y27632, which alone failed to affect the Akt phosphorylation (Fig. 4A, upper panel). Pretreatment with 1 μM of Y27632 caused ~60% increase (Fig. 4A, upper panel, lane 4 compared to lane 2) and 10 μM of Y27632 caused 300% increase (Fig. 4A, upper panel, lane 8 compared to lane 2) in the Akt phosphorylation level. In a similar way, when the cells were pretreated with fasudil, the phosphorylation level of Akt was also dose-dependently enhanced in the range between 3, and 10 μM (Fig. 4B, upper panel).

Effects of Y27632 or fasudil on the EGF-induced phosphorylation of GSK-3β in SW480 colon cancer cells. GSK-3ß is a critical downstream element of the PI3K/Akt cell survival pathway, and its activity can be inhibited by Akt-mediated phosphorylation of GSK-3ß (9,26). Therefore, we next examined the effect of Y27632 or fasudil on phosphorylation of GSK-3ß. We found that EGF markedly induced the phosphorylation of GSK-3ß in a time-dependent manner (data not shown) as reported previously (27). Y27632 increased the EGF-induced phosphorylation level of GSK-3ß (Fig. 5A). Pretreatment with 3 μM of Y27632 caused ~120% increase (Fig. 5A, upper panel, lane 6 compared to lane 2) and 10 μM of Y27632 caused 250% increase (Fig. 5A, upper panel, lane 8 compared to lane 2) in the GSK-3ß phosphorylation level. In addition, when the cells were pretreated with fasudil, GSK-3ß phosphorylation level was also dose-dependently enhanced in the range between 1 and 10 μM (Fig. 5B, upper panel). Interestingly, unlike Akt, when the cells were pretreated with Rho-kinase inhibitors, the phosphorylation levels of GSK-3ß were increased even in EGF-unstimulated cells (Fig. 5A and B, lanes 3, 5 and 7 compared to lane 1, respectively).

Effects of Y27632 or fasudil on the cyclin D1 expression level and Rb phosphorylation induced by EGF in SW480 colon cancer cells. As described above, the Akt/GSK-3ß signaling pathway is closely related to the expression of cyclin D1 and phosphorylation of Rb (28). We next examined the effect of EGF on cyclin D1 expression level and Rb phosphorylation in SW480 cells. As shown in Fig. 6, EGF caused significant increase in both of cyclin D1 expression level and phosphorylation level of Rb (Fig. 6A, B and C, lane 2 compared to lane 1, respectively). Interestingly, pretreatment with Y27632 significantly enhanced the EGF-induced cyclin D1 expression level at a dose of 10 μM (Fig. 6A, upper panel; 6B, lane 6 compared to lane 2). Concurrently, phosphorylation level of Rb was also enhanced in the cells pretreated with Y27632 (Fig. 6A, middle panel; 6C, lane 6 compared to lane 2). Moreover, phosphorylation level of Rb as well as cyclin D1 expression level was increased even in EGF-unstimulated cells when the cells were pretreated with Y27632 (Fig. 6A, B and C, lanes 3 and 5 compared to lane 1), indicating that

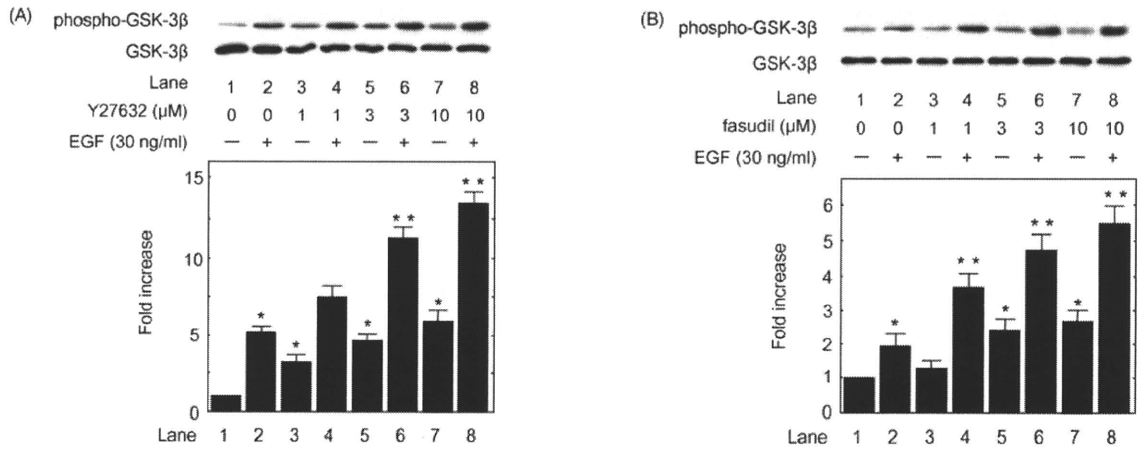

Figure 5. Effects of Rho-kinase inhibitors on EGF-induced phosphorylation of GSK-3ß in SW480 colon cancer cells. The cells were pretreated with the indicated doses of Y27632 (A) or fasudil (B) for 60 min, and then stimulated with 30 ng/ml EGF or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against phospho-specific GSK-3ß or GSK-3ß. The lower bar graph shows the quantification data for the relative levels of phospho-GSK-3ß, after normalization with respect to total GSK-3ß, as determined by densitometry. (*) indicates significant increase ($p<0.05$), as compared with the control (lane 1), respectively. (**) indicates significant increase ($p<0.05$), as compared with the control (lane 2), respectively.

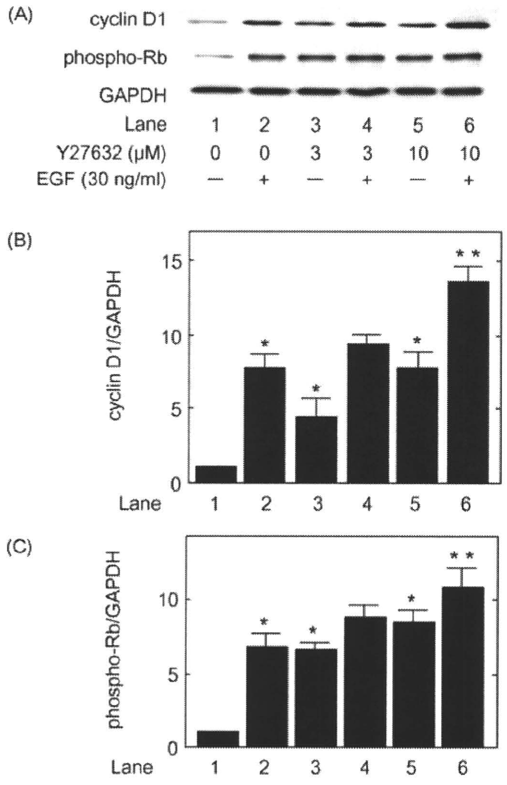

Figure 6. Effects of Rho-kinase inhibitors on EGF-induced expression of cyclin D1 and phosphorylation of Rb in SW480 colon cancer cells. (A) SW480 cells were pretreated with the indicated doses of Y27632 for 60 min, and then stimulated with 30 ng/ml EGF or vehicle for 24 h. The extracts of cells were subjected to SDS-PAGE with subsequent Western blot analysis with antibodies against cyclin D1, phospho-specific Rb and GAPDH. (B and C) The bar graph shows the quantification data for the relative levels of cyclin D1 (B) and phospho-Rb (C), respectively, after normalization with respect to GAPDH, as determined by densitometry. (*) indicates significant increase ($p<0.05$), as compared with the control (lane 1), respectively. (**) indicates significant increase ($p<0.05$) between the indicated pairs, respectively.

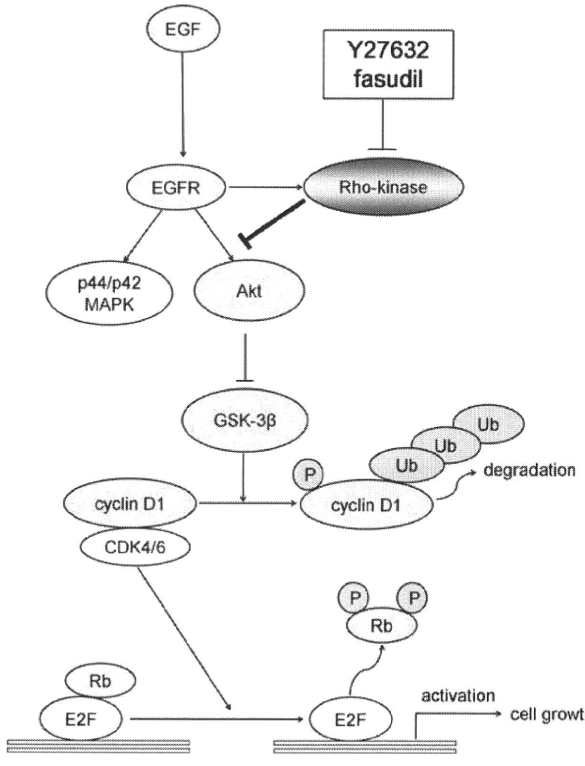

Figure 7. Schematic representation of the involvement of Rho-kinase in the mechanism of EGF-induced cell proliferation in SW480 colon cancer cells. Whereas EGF induces the activation of Rho-kinase as well as p44/p42 MAP kinase and Akt/GSK-3ß, Rho-kinase also functions upstream of Akt/GSK-3ß. Since GSK-3ß is typically active in unstimulated cells, inactivated GSK-3ß stimulated by EGF results in the increased expression level of cyclin D1 followed by Rb phosphorylation, leading to cell growth. Taken together, our results suggest that Rho-kinase regulates negatively EGF-induced cell proliferation in SW480 colon cancer cells. EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, mitogen-activated protein kinase; GSK-3ß, glycogen synthese kinase-3ß; CDK, cyclin-dependent kinase; Rb, retinoblastoma tumor suppressor protein; Ub, ubiquitination.

Y27632 also suppressed the basal activity of Rho-kinase in SW480 colon cancer cells, which is consistent with the results shown in Fig. 2B. Since it is well known that cyclin D1 complex with CDK4/6 causes the phosphorylation and inactivation of Rb (11), resulting in cell proliferation, it is most likely that the activation of Rho-kinase negatively regulates colon cancer cell growth.

Discussion

In the present study, we investigated how Rho-kinase works on cell proliferation of colon cancer cells. First, we examined the effect of Y27632 on cell growth and found that Y27632 alone significantly enhanced cell proliferation in SW480 cells (Fig. 1A and B). In addition, utilizing anti-EGFR-neutralizing antibodies, we found that EGF stimulation is necessary for cell growth in SW480 cells. Therefore, in the following experiments, we examined the involvement of Rho-kinase in EGF signaling in SW480 colon cancer cells. EGF markedly induced the phosphorylation of MYPT-1, known as a downstream substrate of Rho-kinase (5), in a time-dependent manner (Fig. 2A) and Y27632 and fasudil, both of which are specific inhibitors of Rho-kinase (15), clearly suppressed the EGF-induced phosphorylation levels of MYPT-1 (Fig. 2B and C). To confirm these above results, we examined the effect of extracellular EGF on the phosphorylation level of MLC, which is also known to be a downstream target of Rho-kinase (18), by an immunofluorescence study. We showed that EGF induced the phosphorylation of MLC and Y27632 suppressed EGF-induced phosphorylation of MLC (Fig. 2D), suggesting that EGF indeed stimulates the activation of Rho-kinase in colon cancer cells.

To further investigate the involvement of Rho-kinase in the EGF signaling, we next examined the effects of Rho-kinase inhibitors on the EGF-induced activations of p44/p42 MAP kinase and Akt, both of which are major molecules of the signaling cascades in cancer cell proliferation (19). Whereas EGF caused the phosphorylation of both molecules, the phosphorylation of Akt, but not p44/p42 MAP kinase, was surprisingly enhanced when the cells were pretreated with either Y27632 or fasudil (Figs. 3 and 4). Since it is well known that PI3K acts upstream of Akt (7), we further investigated the effects of these inhibitors on activation of PI3K. However, both Y27632 and fasudil have little effect on the phosphorylation of PI3K induced by EGF (data not shown). Taken together, it is most likely that Rho-kinase functions at a point between Akt and PI3K in the EGF signaling, and negatively regulates its signaling in SW480 colon cancer cells.

We also demonstrated that the phosphorylation of GSK-3ß induced by EGF was markedly enhanced when the cells were pretreated with Y27632 and fasudil (Fig. 5). GSK-3ß regulates cyclin D1 proteolysis and subcellular localization, and cyclin D1 complex with CDK4 and CDK6 to stimulate their kinase activities, which in turn cause the phosphorylation and inactivation of Rb, directing these cells toward proliferation (11). Whereas EGF markedly increased the phosphorylation of Rb as well as cyclin D1 protein expression level, pretreatment with Y27632 accelerated them (Fig. 6A), fasudil also enhanced the phosphorylation (data not shown). Taking these findings into account as a whole, our present study suggests that Rho-kinase acts as a negative regulator on colon cancer cell proliferation upstream of Akt/GSK-3ß in the EGF signaling pathway. The potential role of Rho-kinase on EGF-stimulated proliferation of colon cancer cells shown here is summarized in Fig. 7.

Accumulating evidence shows a pivotal role of the Rho/Rho-kinase pathway in the regulator of cytoskeletal dynamics, cell adhesion and cell migration (29), indicating its central role in cancer cell invasion and metastasis. Y27632 reportedly interferes with Ras-mediated transformation, and constitutively active mutants of Rho-kinase, collaborate with activated Raf in transformation assays in NIH3T3 cells (30). On the contrary, it has been shown that inactivation of the Rho/Rho-kinase pathway promotes oncogenic Ras-induced transformation in Rat1 fibroblast cells, suggesting a negative role of Rho-kinase in oncogenic cells that is consistent with our present study (31). Thus, since the precise role of Rho-kinase in cancer progression still remains controversial, further investigation is required to elucidate the exact role of Rho-kinase in colon cancer cell growth.

In conclusion, Rho-kinase regulates negatively EGF-induced cell proliferation upstream of Akt/GSK-3ß in colon cancer cells. This is the first report, which shows that Rho-kinase is involved in negative regulation for EGF-induced cell proliferation, thus indicating important insights into therapeutics for colon cancer. Regulation of Rho-kinase might be considered as a new clinical target for colon cancer patients.

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Hepatology Research 2010; 40: 1–7 doi: 10.1111/j.1872-034X.2009.00633.x

Review Article

Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus infection for the fiscal year 2008 in Japan

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In the 2008 guidelines for the treatment of patients with cirrhosis, who are infected with hepatitis B virus (HBV), the main goal is to normalize levels of alanine and aspartate aminotransferases by eliminating HBV or reducing viral loads. In patients with compensated cirrhosis, the clearance of HBV from serum is aimed for by entecavir, as the main resort, for histological improvement toward the prevention of hepatocellular carcinoma (HCC). In patients with decompensated cirrhosis, by contrast, meticulous therapeutic strategies are adopted for the reversal to compensation, toward the eventual goal of decreasing the risk of HCC. For maintaining liver function and preventing HCC, branched chain amino acids and nutrient supplements are applied, in addition to conventional liver supportive therapies. For patients with chronic hepatitis B, separate guidelines are applied to those younger than 35 years and those aged 35 years or older. Even for patients with chronic hepatitis who are negative for hepatitis e antigen (HBeAg), but who harbor HBV DNA in titers of 7 log copies/mL or more, a "drug-free state" is aimed for by sequential treatment with interferon (IFN) plus entecavir as the first line. For patients with chronic hepatitis B aged 35 years or older, who are HBeAg-negative and carry HBV DNA in titers of less than 7 log copies/mL, long-term IFN for 24–48 weeks is adopted anew. To HBeAg-negative patients who have either or both platelet counts of less than 150×10^3/mm^3 and less than 7 log copies of HBV DNA, also, long-term IFN for 24–48 weeks is indicated.

Key words: chronic hepatitis, cirrhosis, hepatitis B virus, hepatocellular carcinoma, interferon, liver supportive therapies, nucleos(t)ide analogs

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Received 26 October 2009; revision 4 November 2009; accepted 11 November 2009.

INTRODUCTION

SINCE THE FISCAL year 2002, guidelines for the treatment of patients with viral hepatitis have been compiled annually by the Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, under the auspice of the Ministry of Health, Labor and Welfare of Japan, supported by enduring efforts of many specialists recruited from all over the nation. Guidelines have been improved every year with many supplementary issues, which had surfaced as our understanding of many facets of viral hepatitis deepened and treatment options widened increasingly with time. For the fiscal year 2008, guidelines have been worked out for a comprehensive standardization of the treatment of chronic hepatitis and cirrhosis due to hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in Japan. These guidelines have been observed by more than 70% of practicing hepatologists treating patients with viral liver disease in Japan. It is hoped that these guidelines will continue being widely accepted and implemented to help as many patients as possible who are suffering from sequelae of persistent hepatitis virus infections.

Here, we relate excerpts of the 2008 guidelines for the treatment of patients with liver disease due to HBV, covering a wide range from those with chronic hepatitis to those with decompensated cirrhosis. The 2008 guidelines for the treatment of liver disease due to HCV are reported in an accompanying paper.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B

PATIENTS WITH CHRONIC hepatitis B can stabilize the activity of liver disease in their natural course, after they have seroconverted from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe), accompanied by decrease in HBV DNA titers. For that reason, treatment guidelines were constructed separately for the patients younger than 35 years and those aged 35 years or older.

GUIDELINES FOR THE TREATMENT OF PATIENTS WITH CHRONIC HEPATITIS B YOUNGER THAN 35 YEARS

PATIENTS WITH CHRONIC hepatitis B younger than 35 years are treated in accordance with the guidelines summarized in Table 1. Criteria for the treatment eligibility are: (i) serum levels of alanine aminotransferase (ALT) of 31 IU/L or more; and (ii) HBV DNA titers of 5 log copies of more in HBeAg-positive patients and 4 log copies or more in HBeAg-negative patients. In the 2008 guidelines, the indication of treatment is extended to the patients with cirrhosis due to HBV who carry HBV DNA in titers of 3 log copies/mL or more.

In Japan, most HBeAg-positive patients with 7 log copies or more of HBV DNA have been infected with HBV of genotype C by perinatal infection at birth;

Table 1 Guidelines for the treatment of patients with chronic hepatitis B younger than 35 years

Eligibility criteria	ALT	≥31 IU/L
	HBV DNA	HBeAg-positive patients: ≥5 log copies/mL HBeAg-negative patients: ≥4 log copies/mL Patients with cirrhosis: ≥3 log copies/mL
HBV DNA	≥7 log copies/mL	<7 log copies/mL
HBeAg-positive	(1) Long-term IFN for 24–48 weeks (2) Entecavir	(1) Long-term IFN for 24–48 weeks (2) Entecavir
HBeAg-negative	(1) Sequential treatment† (entecavir plus IFN) (2) Entecavir	(1) Regular follow up (2) Long-term IFN for 24 weeks
	Start with entecavir in HBeAg-negative patients who have platelet counts $<15 \times 10^3/mm^3$ and in those with advanced liver disease of stage F2 or higher.	

†Sequential treatment: patients who have lost hepatitis B virus (HBV) DNA after treatment with nucleos(t)ide analogs receive combined interferon (IFN) for 4 weeks, and then IFN monotherapy is continued for 20 weeks, and lifted thereafter. ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen.