Table 5 continued

		Baseline	Post-treatment	*P* value
P value				
Number of patients		0.322	0.322	
Number of lesions		0.314	0.077	
Second tertile				
L. casei group ($n = 13$)				
Number of patients (%)		10 (76.9)	9 (69.2)	>0.999
Total number of lesions	(mean ± SD)	46 (3.5 ± 3.6)	15 (1.6 ± 1.1)	0.118
Median number (range)		2 (0–10)	1 (0–3)	
Control group ($n = 12$)				
Number of patients (%)		9 (75.0)	7 (58.3)	0.667
Total number of lesions	(mean ± SD)	32 (2.7 ± 2.8)	21 (1.8 ± 2.9)	0.273
Median number (range)		1.5 (0–9)	1 (0–10)	
P value				
Number of patients		>0.999	0.688	
Number of lesions		0.664	0.828	
Third tertile				
L. casei group ($n = 13$)				
Number of patients (%)		9 (69.2)	4 (30.8)	0.115
Total number of lesions	(mean ± SD)	35 (2.7 ± 3.3)	10 (0.8 ± 1.4)	0.077
Median number (range)		1 (0–9)	0 (0–4)	
Control group ($n = 12$)				
Number of patients (%)		6 (50.0)	6 (50.0)	>0.999
Total number of lesions	(mean ± SD)	20 (1.7 ± 2.7)	21 (1.8 ± 2.1)	0.795
Median number (range)		0.5 (0–9)	1 (0–6)	
P value				
Number of patients		0.428	0.428	
Number of lesions		0.384	0.289	

The small bowel was divided into three parts (first, second and third tertile) on the basis of each patient's small bowel transit time

small bowel injury in rats and that its probiotic effects may be mediated through the anti-inflammatory effects of lactic acid. More recently, Montalto et al. [20] showed that treatment with probiotic mixture (VSL#3) including *L. casei* significantly reduced the fecal calprotectin concentrations in healthy volunteers receiving indomethacin. However, the efficacy of probiotics has only been indirectly evaluated, and up till now, there have been no reports on low-dose aspirin-associated small bowel injury. In the present study, we directly evaluated the effect of *L. casei* using CE and found that the probiotic stimulated healing of aspirin-associated mucosal injuries.

As regards doses and time of administration, up till the present no clear data regarding the relationship between the amount of probiotic bacteria and the beneficial effects have been reported. In particular, no studies have appeared small bowel injury in chronic NSAIDs/aspirin users. Therefore, we based the therapeutic term (3 months) according to the previous studies [36, 37] in which the effect of the probiotic on small bowel inflammation was endoscopically evaluated in a similar manner to our study. As for the dose of *L. casei*, it would be desirable to use a high dosage probiotics containing a high concentration of live bacteria to ensure their survival with functional activity along the entire length of the intestine. It is unclear whether the dose of *L. casei* used in this study is optimal, but the dose of *L. casei* used in the present study (45×10^8 to 63×10^9 CFU daily) is higher compared with similar previous work. Thus, we designed our study based on the speculation that this dose of *L. casei* would be enough to prevent aspirin-induced small bowel injury. Further investigations are needed to confirm the optimal therapeutic term and doses of probiotic treatment on small bowel injury.

As for the prevention of NSAID-induced small bowel injury, several studies have already shown that omeprazole, a proton pump inhibitor, is not effective [38, 39], whereas misoprostol, a prostaglandin analog, effectively reduced the incidence of small bowel lesions induced by 2 weeks' administration of diclofenac [21]. Prostaglandin has been

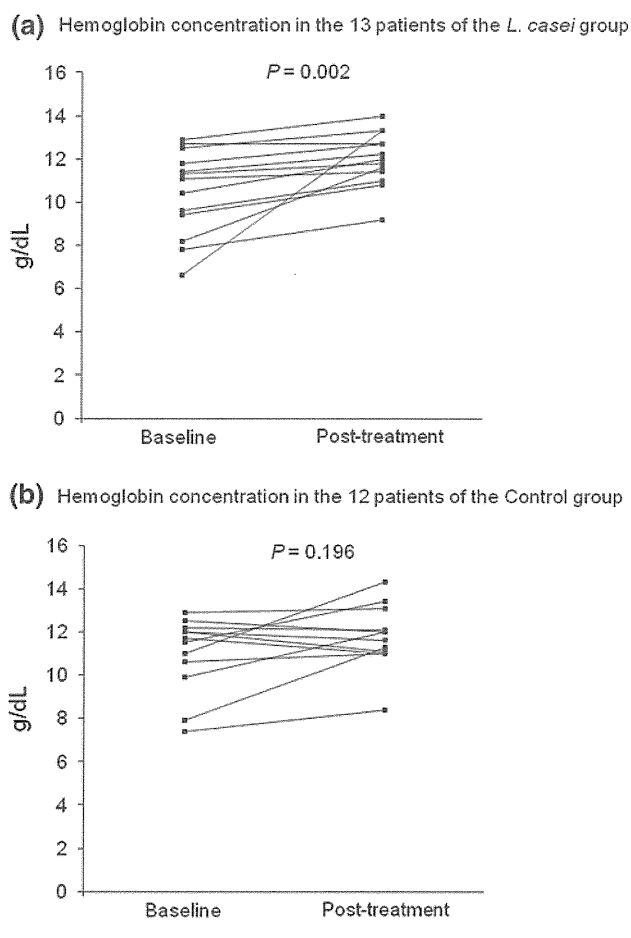

Fig. 4 Hemoglobin concentration at the time of the baseline capsule endoscopy and at the time of the post-treatment capsule endoscopy in the 13 patients of the *L. casei* (**a**) and 12 patients of the control group (**b**)

shown to reverse NSAID-induced changes in intestinal permeability [40], a local intestinal event that is considered to play a pivotal role in the development of inflammation and injury. The efficacy of this drug has also been reported in aspirin-induced enteropathy [41]. However, misoprostol is often poorly tolerated because of its side effects, such as diarrhea and abdominal pain. Indeed, in a pilot study to evaluate the efficacy of misoprostol on aspirin-induced enteropathy, 3 of the 11 patients who received misoprostol discontinued the drug owing to the development of severe watery diarrhea [41]. Therefore, development of an effective alternative agent against NSAID/aspirin-induced enteropathy is strongly desired.

Although our previous report showing the characteristics of small bowel injury in chronic low-dose aspirin users demonstrated that ulcers were observed mainly in the distal part of the small bowel [9], the mucosal breaks in this study did not show a similar tendency. A possible reason for this discrepancy is that the definition of the CE findings in this study abandoned the differentiation of mucosal breaks from other terms, such as erosions or ulcers, to simplify the evaluation of the efficacy of *L. casei* on aspirin-induced small bowel mucosal injury. Indeed, ulcers showed a tendency to exist in the distal part of the small bowel if we differentiated ulcers from mucosal breaks in this study (data not shown). Another possible reason is the interindividual differences among the patients who participated in the study. This study included asymptomatic patients with unexplained iron deficiency anemia, while our previous study included symptomatic patients with symptoms such as gastrointestinal bleeding or abdominal pain [9]. Furthermore, we compared the distribution of small bowel injuries at the baseline and post-treatment CE to evaluate the correlation between the therapeutic effect of *L. casei* and the distribution of aspirin-induced small bowel injuries. In the first tertile, significant decreases in the percentage of the patients with mucosal breaks and the number of mucosal breaks were observed at the post-treatment CE compared with the results at the baseline CE. A decrease in the number of mucosal breaks in the third tertile was also observed in response to probiotic treatment in the *L. casei* group; however, the difference did not reach statistical significance. These results may be influenced by the difference in the intestinal microbial flora between the proximal and the distal small bowel [42]. The luminal bacterial load increases from the proximal to the distal small bowel, and these changes may play a pathogenic role in NSAID/aspirin-induced injury. The efficacy of probiotic treatment in the distal part of the small bowel can be explained by the modulation of the abundant intestinal bacteria, thereby preventing enterobacteria from invading the small bowel mucosa. Although the intestinal bacterial flora is likely to be sparse in the proximal small bowel, low numbers of microorganisms, mainly consisting of acid-tolerant lactobacilli and streptococci, exist in the proximal part of the small bowel. The probiotics might have a great effect on these enterobacteria in the proximal small bowel because the concentration of ingested live bacteria with functional activity is higher in the proximal small bowel than in the distal small bowel. The actual mechanisms of probiotics against small bowel injuries remain poorly understood, and further investigations are needed.

CE has revealed numerous inflammatory lesions and has shed light on the small bowel mucosal injury induced by NSAIDs and aspirin. Despite these investigations, the clinical significance of NSAID/aspirin-associated mucosal injury is not yet clear. Almost all the patients taking low-dose aspirin have some degree of intestinal mucosal injuries, but it has not been investigated as to whether these lesions of the small bowel can actually explain the iron deficiency anemia of unknown source in patients on low-dose aspirin. Our results demonstrated that treatment with *L. casei* produced a significant improvement in serum hemoglobin concentration

that was not observed in the control individuals. The hemoglobin concentration in the *L. casei* group changed in parallel with the small bowel mucosal injuries (the number of small bowel lesions and CE score), suggesting that these mucosal injuries might induce microbleeding and be the cause of the anemia of unknown source. On the other hand, the hemoglobin concentrations in a few patients in the control group increased without probiotic treatment. Further studies are needed to elucidate the correlation between the small bowel injuries and changes in the blood hemoglobin concentration in chronic low-dose aspirin users.

The present study had a number of limitations. The primary concern is the possibility that the CE findings might not be direct consequences of the low-dose aspirin administration. Follow-up CE examinations after aspirin withdrawal were not performed because the majority of the patients who take aspirin as an antiplatelet agent could not discontinue it. However, no patients had a new diagnosis of Crohn's disease, Behçet's disease, intestinal tuberculosis or other inflammatory bowel diseases. Moreover, the CE findings and scores in this study were consistent with those in other recent investigations that studied the characteristics of the small bowel injury in chronic low-dose aspirin users [9, 23, 41]. Thus, although most of the CE findings of this study are suggestive, they are still not definitive. Another limitation was the design of this study. Although our study was conducted as a randomized controlled trial, there was no placebo control group, and the study size was small. A placebo-controlled large-scale trial is needed to confirm our results. In addition, four patients were excluded from the current analysis after the randomization, and the follow-up CE was not performed in these patients. An intention-to-treat analysis would be desirable.

In conclusion, data from this pilot study suggest that probiotic treatment (*L. casei*) protects against aspirin-associated small bowel injury. Further larger scale studies are necessary to confirm the beneficial effect of probiotics.

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Conflict of interest The authors of the article have nothing to disclose.

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Optimal Approach for Small Bowel Capsule Endoscopy Using Polyethylene Glycol and Metoclopramide with the Assistance of a Real-Time Viewer

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Key Words
Capsule endoscopy · Polyethylene glycol · Metoclopramide · Real-time viewer

Abstract
Aim: Capsule endoscopy is limited by the poor image quality of the distal bowel and incomplete small bowel transit. The aim of this study was to establish an optimal medication protocol for capsule endoscopy performed using a real-time viewer. ***Methods:*** A total of 80 patients were prospectively recruited. The patients were randomized into two groups: the 'conventional group' (without any preparation) and the 'real-time group' (in which a real-time viewer was attached). At 60 min after swallowing the capsule, if the capsule had reached the small bowel, 500 ml of polyethylene glycol was administered; if the capsule was still located in the stomach, 10 mg of metoclopramide was given intramuscularly, followed by 500 ml of polyethylene glycol solution. ***Results:*** The completion rate was significantly higher in the real-time group as compared with that in the conventional group (72.5 vs. 90.0%). Our protocol yielded a significantly improved image quality of the distal small bowel [image quality score = 1.6 vs. 3.0 (max 4.0)]. The detection rate of lesions in the distal small bowel was higher in the real-time group than in the conventional group. ***Conclusions:*** The present study clearly showed that our protocol yielded an improved completion rate and also improved image quality.

Introduction

Capsule endoscopy (CE) has been developed as a convenient method for evaluation of the small bowel. CE provides a higher diagnostic yield than barium contrast radiography of the small bowel or enteroscopy [1–4]. It is safe, painless, and well tolerated [5]. Despite its advantages, the diagnostic yield of CE may be restricted by some limitations. In almost 20–30% of the cases, the capsule does not reach the cecum within the imaging period [6–8]. This results in failure of visualization of the more

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distal small bowel. In addition, the overall results differ among studies, with the reported percentage of cases with incomplete visualization of the mucosal surfaces due to bubbles, or luminal residue obscuring the view, especially in the distal small bowel, varying from 5 to 30% [9–12]. Several studies have examined the possibility of shortening the transit time and improving the bowel cleanness using different medications for bowel preparation and prescribing different fasting periods [13, 14]. Nevertheless, small bowel preparation still remains a controversial issue. The optimal method of preparation and the dose/time of administration of the agents used still remain to be determined.

Recently, external real-time viewers have been developed by some companies, including Given Imaging Ltd (Yoqneam, Israel) and Olympus Co. (Tokyo, Japan). This device enables real-time viewing by the physician performing the CE procedure. The main purpose of this device is to detect impaired gastric emptying or any cause of blockage of the capsule [15]. However, the clinical usefulness of a real-time viewer in CE remains unknown. The aim of this study was to establish an optimal medication protocol for CE performed using real-time viewer, in order to improve the CE completion rate and the image quality.

Methods

Patients

The study was performed between May 2009 and April 2010 at Yokohama City University School of Medicine. A total of 80 subjects who were judged, based on the history and physical examination, to be in acceptable health without severe systemic diseases that could affect a CE examination were enrolled in this study. The exclusion criteria were as follows: history of gastric or intestinal surgery, clinical or suspected abnormalities in gastric emptying, pregnancy, age <18 years, and intake of medications during the previous week that could potentially affect the gastrointestinal motility. The study was carried out in accordance with the Declaration of Helsinki (revised 1995), and was approved by the Ethics Committee of Yokohama City University School of Medicine. Written informed consent was obtained from each of the patients for the CE study and also for the use of the data and images for research purposes.

Study Design

In all the patients, the CE was performed with the PillCam SB and SB2 capsule endoscopy system (Given Imaging Ltd), and the images were viewed with the Rapid 5 Reader. Patients were randomized into two groups through the use of sealed envelopes. In the first conventional CE group, the patients were instructed to fast for 12 h prior to the CE procedure, and swallow the capsule with water and 0.5 ml simethicone. In the real-time group, pre-procedure preparation was carried out as described above for the conventional CE group, plus a real-time viewer was attached to the patients. At 60 min after it was swallowed, if the capsule had reached the small bowel, 500 ml of polyethylene glycol (PEG), as a purgative, was administered to the patient. If the capsule was still located in the stomach, 10 mg of metoclopramide, as a prokinetic agent, was given intramuscularly, followed by 500 ml of oral PEG (fig. 1).

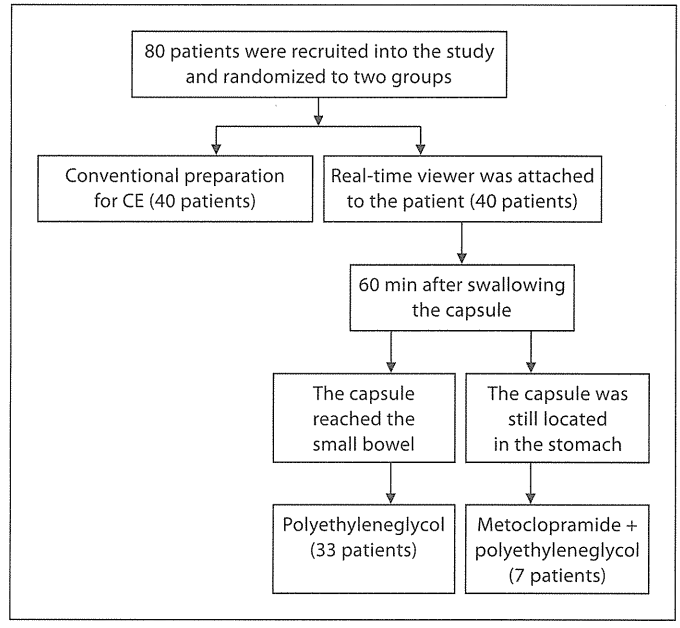

Fig. 1. Flow diagram of the study.

Patients of both groups were permitted to drink fluids at 2 h and to eat a meal at 4 h after the capsule ingestion. After 8 h, they returned to the endoscopy unit where the recorder was removed and the images were downloaded.

Scoring System

All CE examinations were read by two investigators (H.E. and K.H.). Both were blinded to the group allocation status of the patients. Small bowel examination was considered to be complete if the capsule had passed into the colon. The gastric transit time (GTT) was calculated from the time the capsule entered the stomach until it passed the pylorus. Small bowel transit time (SBTT) was determined as the time from the first duodenal image until the capsule entered the colon, and could be calculated only in cases in which the capsule reached the colon.

The quality assessment of the capsule endoscopic image was in accordance with the scale used by Aymer et al., with some modification [16]. The image quality was evaluated only in cases in which the capsule reached the colon. We used a 5-point scale (0–4) based on the percentage of the capsule images that was unimpaired by the presence of debris or dark luminal fluid (4, 100–80%; 3, 80–60%; 2, 60–40%; 1, 40–20%; 0, 20–0%) (fig. 2). The average score for 5-min segments of the video was assessed from capsule entry into the proximal duodenum (0% of the SBTT), and evenly spaced for every 10% of the SBTT thereafter, with the final segment recorded in the terminal ileum (100% of the SBTT). For

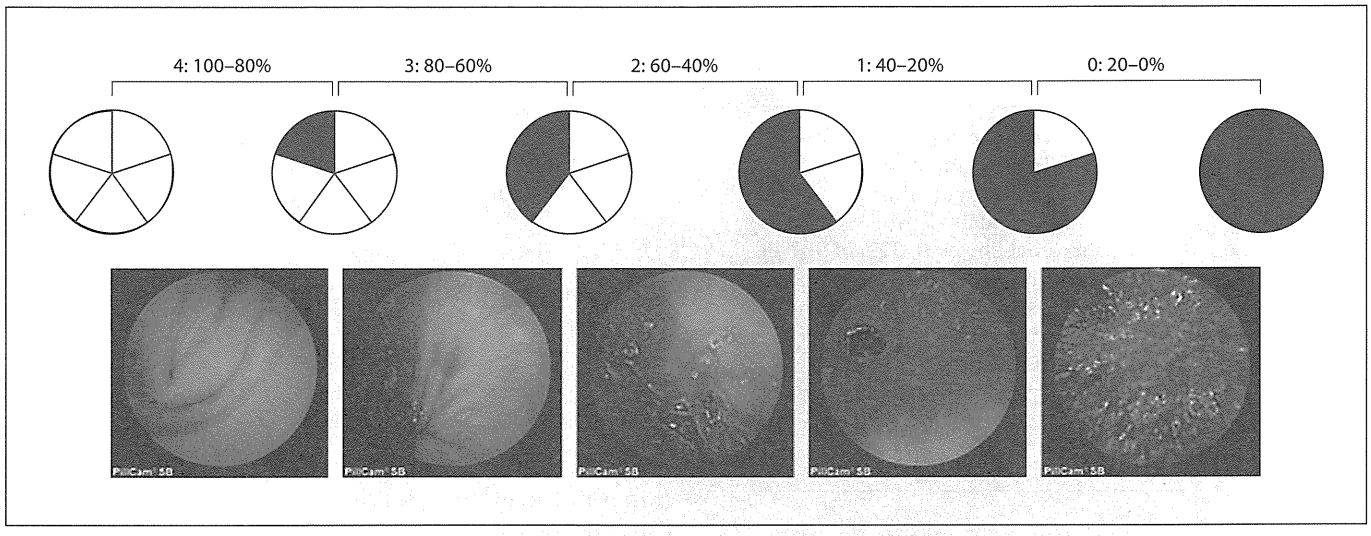

Fig. 2. Image quality score: score based on the percentage of the capsule endoscopic images that were unimpaired by debris or dark luminal fluid: 4, 100–80%; 3, 80–60%; 2, 60–40%; 1, 40–20%; 0, 20–0%.

Table 1. Characteristics of the patients undergoing conventional and real-time CE

	Conventional	Real-time	p values
Patients, n	40	40	
Age (± SD), years	58.5 ± 15.8	60.6 ± 16.5	0.56
Male, n	23 (57.5%)	27 (67.5%)	0.36
Body mass index (± SD)	21.6 ± 3.1	21.5 ± 3.6	0.87
Diabetes mellitus, n	6 (15.0%)	5 (12.5%)	0.74
Hospitalization, n	9 (22.5%)	9 (22.5%)	–
Indication for CE, n			
OGIB and/or IDA	31 (77.5%)	35 (87.5%)	0.24
Abdominal pain	3 (7.5%)	2 (5.0%)	0.64
Others	6 (15.0%)	3 (7.5%)	0.29

OGIB = Obscure GI bleeding; IDA = iron deficiency anemia.

the subsequent statistical analysis, the 5-min segments were also combined to allow an assessment of the bowel cleanness in the proximal third (0, 10, 20, and 30% of the SBTT), middle third (40, 50, and 60 of the SBTT), and distal third of the small bowel (70, 80, 90, and 100% of the SBTT).

Statistical Analysis

The results were presented as mean ± SD for the quantitative data and as frequency (percentage) for the categorical data. Data with a normal distribution were compared using the two-sided Student's t test. Categorical data were analyzed using the χ^2 test or the Yates χ^2 test. p values <0.05 were considered to denote statistical significance. To assess the reporting consistency between the two reviewers for image quality, the κ score was calculated.

Results

Characteristics of the Patients and the Indications for CE

A total of 80 patients were prospectively recruited for this study within the defined periods. The characteristics of the patients and the indications for CE are shown in table 1. Forty patients were randomized to the conventional CE group, and the remaining patients underwent real-time CE. The mean age of patients was 59.8 years (range 21–84), and 62.5% were male. The most frequent indication for CE was obscure gastrointestinal bleeding (82.5%). There was no significant difference in age (p = 0.56), sex ratio (p = 0.36), body mass index (p = 0.87), prevalence of diabetes mellitus (p = 0.74), frequency of hospitalization, or distribution of the indications for CE between the two groups.

Transit Times, Completion Rate and Adverse Events

The small bowel examination could be completed in 29 patients of the conventional CE group (72.5%) and 36 patients of the real-time CE group (90.0%) (p = 0.04). The mean GTT was 42.0 ± 41.4 and 38.7 ± 32.9 min in the conventional and real-time CE groups, respectively (±SD, p = 0.69). The mean SBTT was 278.4 ± 76.5 and 235.0 ± 116.2 min in the conventional and real-time CE groups, respectively (p = 0.08) (table 2). At 60 min after it had been swallowed, there were 8 and 7 patients in the conventional and real-time groups, respectively, in whom

Table 2. Completion rate, GTT, SBTT, and image quality in the study groups

	Conventional	Real-time	p values
Completion rate, n (%)	29/40 (72.5)	36/40 (90.0)	0.04*
Mean GTT (range), min	42.0 (1–158)	38.7 (4–141)	0.69
Mean SBTT (range), min	278.4 (91–414)	235.0 (20–428)	0.08
Mean image quality score			
Proximal bowel (0–30% of SBTT)	3.2	3.4	0.37
Mid small bowel (40–60% of SBTT)	2.5	3.2	0.055
Distal small bowel (70–100% of SBTT)	1.6	3.0	<0.001*

* $p < 0.05$.

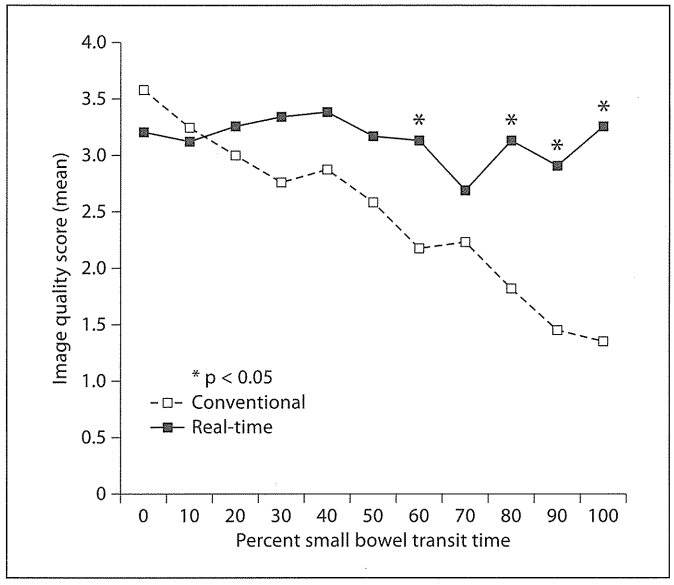

Fig. 3. Image quality score according to the extent of capsule progression through the small bowel. In the conventional group, the image quality worsened as the capsule progressed distally. On the other hand, the image quality tended to be better in the real-time CE group than in the conventional CE group for all small bowel segments.

the capsule was still located in the stomach. Therefore, metoclopramide was used as a prokinetic agent in these 7 patients of the real-time CE group. In 6 of these 7 patients (85.7%: 6/7), the capsule then passed into the cecum within the recording time. The mean GTT and SBTT in the 6 patients in whom total enteroscopy could be completed using metoclopramide were 94.3 ±17.6 and 201.8 ± 157.1 min, respectively. The GTT in the remaining 1 patient in whom successful execution of total enteroscopy failed even after metoclopramide administration was 141 min. On the other hand, in the conventional CE group, the capsule reached the cecum within the recording time in only 4 of the 8 patients (50%), in all of whom the GTT exceeded 60 min. The mean GTT and SBTT in the remaining 4 patients of the group in whom total enteroscopy could be accomplished were 103.8 ± 23.4 and 218.0 ± 86.1 min, respectively. The GTT in the 4 patients of the group with failed total enteroscopy was 122.5 ± 43.7 min.

There were no known cases of capsule retention or serious adverse events in any of the study participants. In the real-time CE group, none of the patients complained of discomfort or difficulty in ingesting 500 ml of PEG solution.

Image Quality

The image quality was good in the proximal bowel segment in both groups. The mean image quality scores were 3.2 and 3.4 in the conventional and real-time CE groups, respectively ($p = 0.37$) (table 2). However, in the conventional CE group, the image quality became worse as the capsule progressed more distally. In the mid small bowel segment, the mean image quality scores were 2.5 and 3.2 in the conventional and real-time CE groups, respectively ($p = 0.055$). The views were impaired by bile, residue, and bubble artifacts. On the other hand, in the real-time group, the image quality scores tended to be better than those in the conventional CE group for all the small bowel segments. There was a significant difference between the two groups with regard to the image quality for the distal small bowel (1.6 in the conventional CE group vs. 3.0 in the real-time CE group, $p < 0.001$) (fig. 3). The κ statistic (between authors H.E. and K.H.) demonstrated a good level of consistency, with a value of 0.71 (95% CI, 0.51–0.86).

Positive Findings and Diagnostic Yield

Positive findings were obtained in 25 of the 40 (66.5%) patients in the conventional CE group and in 29 of the 40 (72.5%) patients in the real-time CE group (table 3). Positive findings included angioectasia, ulcers, erosions, and tumors/polyps. The diagnostic yield in the conventional group was lower than that in the real-time group; however, the difference did not reach statistical significance ($p = 0.34$). However, when the lesion detection rate was compared according to the segment of the small bowel, a larger number of lesions was detected in the real-time CE group as compared with that in the conventional CE group in the distal third of the small bowel ($p = 0.003$) (fig. 4).

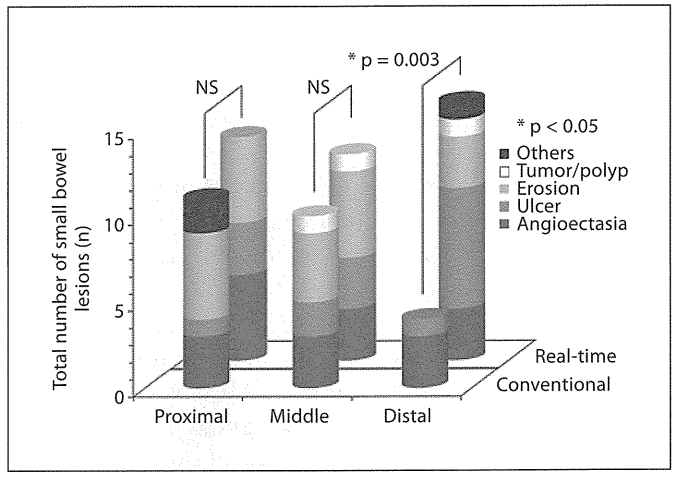

Fig. 4. Relationship between the distribution of small bowel lesions and the allocated group of the patients (conventional CE, real-time CE group). In the distal third of the small bowel, there were more numerous lesions in the real-time group than in the conventional group.

Table 3. Diagnostic yield and number of patients with positive findings

	Conventional	Real-time	p values
Diagnostic yield, n (%)	25/40 (66.5)	29/40 (72.5)	0.34
Patients with positive findings, n			
Angioectasia	8	8	–
Ulcer	4	10	0.07
Erosion	9	9	–
Tumor/polyp	1	1	–
Others	3	1	0.31
No findings	15	11	0.26

Discussion

In recent years, the indications for CE examinations have expanded. CE is expensive and time-consuming, and in order to reduce the number of unfruitful procedures and minimize the cost, it is necessary to establish a protocol for increasing the diagnostic yield. The diagnostic yield of CE depends on several factors, including the GTT, SBTT, and the image quality [17]. The capsule passage time varies considerably among subjects because the capsule is transported passively through the gastrointestinal tract. If the capsule is transported slowly and does not reach the cecum within the recording time, the CE would be incomplete. In cases of incomplete CE, significant lesions outside the observation range may be missed. For example, small bowel ulcers in chronic low-dose aspirin users tend to exist in the distal part of the small bowel [18]. Incomplete CE would be associated with a lower likelihood of visualization of these lesions, making the procedure wasteful. Moreover, in the distal segment of the small bowel, the presence of luminal residue worsens the quality of the images [9–12]. If the quality of the image is poor, the lesions can be masked by the bowel contents.

Recently, a new generation of CE has been equipped with a real-time video monitor that enables real-time visualization of the small bowel lumen. Some studies have demonstrated the usefulness of a real-time viewer; however, its role and significance have not yet been clarified. Lai et al. [19] used real-time viewing to monitor the capsule progression. If the capsule had not reached the small bowel by the first 30 min, an additional liter of PEG was given to the patient. If the capsule still failed to enter the small bowel within the next 30 min, 250 mg of erythromycin was given orally to accelerate gastric emptying. They found that their interventions might have helped to improve the CE completion rate. Ogata et al. [20] also demonstrated that the real-time viewer was useful for detecting gastric transit abnormalities and determining the need for metoclopramide administration in patients undergoing CE. These reports evaluated mostly the CE completion rate, but did not shed any light on the improvement of the image quality. To gain good image quality, an optimal medication protocol should be designed.

In this study, according to the progress of the capsule as viewed by the real-time viewer, we prescribed appropriate medications, such as a purgative or a prokinetic

agent. The results of the study show that our protocol increased the CE completion rate and also improved image quality, especially in the distal small bowel. As a result, the lesion detection rate in the distal small bowel increased.

In order to increase the CE completion rate, we used metoclopramide as a prokinetic agent. It has been noted that prolonged capsule retention within the stomach reduces the likelihood of complete small bowel transit within the recording time [21]. It has been reported that the use of prokinetics such as metoclopramide [22], erythromycin [14] and mosapride [23] may decrease the randomness of the gastric emptying and reduce the SBTT. Selby [22] reported that the administration of oral metoclopramide before the capsule ingestion reduced the GTT. Metoclopramide has several actions that may account for its favorable influence on the capsule transit time. Its main effect is in the proximal gastrointestinal tract. It improves the gastric tone and peristalsis, relaxes the pyloric sphincter, and improves antroduodenal coordination [24] by a combination of its cholinergic and antidopaminergic effects [25]. It also increases the motility of the proximal small bowel [26]. In the present study, we prescribed metoclopramide in cases where the capsule was retained within the stomach for >60 min, as assessed with the assistance of the real-time viewer. In this study, the time was set at 60 min because the third quartile value of the GTT without the use of any prokinetic agents or bowel preparations was determined previously at our department to be approximately 60 min (data not shown).

In order to improve the image quality, we used a small volume of PEG solution as a purgative. Some studies have demonstrated that adequate bowel preparation can lead to improvement of the small bowel image quality and thereby increase the diagnostic yield [27]. For example, Viazis et al. [13] demonstrated that administration of 2 l of a PEG solution, as a bowel preparation agent, 16 h before performing CE, increased the diagnostic yield. Ingestion by the patient of 4 l of a PEG solution was also reported to improve the quality of visualization of the small bowel and the complete CE rate [10]. The main disadvantage, however, of bowel preparation is that it is not comfortable for the patients [28], and it has been revealed as a major source of discomfort in patients undergoing colonoscopy. It would be a considerable burden for patients to drink large amounts of PEG solution, and smaller volumes may be more acceptable. Therefore, in the present study, 500 ml of PEG solution was used for bowel preparation. Indeed, none of the patients complained of discomfort or difficulty in ingesting the PEG solution, and this volume appeared to be sufficient to clean the distal small bowel.

The present study had some limitations. First, the number of study subjects was small, although statistically significant differences in the CE completion rate and image quality between the two study groups were achieved. Second, the decision to use 500 ml of PEG solution about 60 min after ingestion of the capsule was not based on real-time observation of the capsule position. The timing of administration of PEG solution is important. Some investigators have suggested that PEG may increase the GTT [11, 29]. Ben-Soussan et al. [11] mentioned that a hyperosmolar state was generated by the PEG preparation, leading to increased gastric distension with fluid and decreased release of the gastric contents. To avoid these effects, it would be desirable to have the capsule already in the small bowel when the PEG is administered. However, it is difficult to continually monitor the progress of the capsule through the stomach into the small bowel by real-time viewing. Therefore, we used metoclopramide when we detected delayed gastric emptying of the capsule with the real-time viewer, in the hope that the capsule would reach the small bowel early.

In conclusion, the results of the present study clearly showed that our protocol using PEG and metoclopramide improved the CE completion rate and image quality. Therefore, our protocol may be useful in clinical practice for CE.

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Leptin acts as a growth factor for colorectal tumours at stages subsequent to tumour initiation in murine colon carcinogenesis

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ABSTRACT

Background and aims Obesity increases the risk of colorectal cancer (CRC). Serum leptin levels are markedly elevated in obese individuals, but the involvement of leptin in CRC growth remains unclear. We explored the hypothesis that leptin signalling regulates the growth of CRC, by examining the effects of leptin deficiency on murine colon tumour growth.

Methods We used genetic (leptin-deficient and leptin receptor-deficient) models of obesity and investigated carcinogen-induced colon polyp formation and cell proliferation in the colonic epithelium. Colonic tissues and cell lines were analysed by histopathology and molecular-biology methods.

Results A significant increase in the proliferative activity of normal colonic epithelial cells was observed in the obesity model; on the other hand, significant decrease of tumour cell proliferation was observed in leptin-deficient tumours, and tumour growth was dramatically inhibited in leptin-deficient and leptin-receptor-deficient mice despite the animals exhibiting severe obesity. Notably, a marked increase of the leptin receptor (ObR) expression levels was observed in colon tumours as compared to the normal epithelium. Nuclear β-catenin staining was pronounced in all tumours, irrespective of leptin deficiency, whereas altered cellular localisation of β-catenin was not observed in the normal colonic epithelial cells. In vitro, β-catenin knockdown decreased ObR expression, and stimulation of recombinant Wnt increased ObR expression. In addition, the proliferative and survival effects of leptin were found to be mediated by the ObR/signal transducer and activator of transcription 3 (STAT3) signalling in colon tumours.

Conclusions Our findings indicate that leptin is important for CRC growth in obesity, and acts as a growth factor for CRC at stages subsequent to tumour initiation in colorectal carcinogenesis. Thus, inhibition of leptin signalling may be an effective strategy for therapy and prevention of colonic adenoma and cancer, which show activation of Wnt signalling.

Significance of this study

What is already known about this subject?

- Epidemiological studies have revealed that obesity raises the risk of colon adenoma and colorectal cancer (CRC), and the results of animal experiments suggest a link between obesity and CRC.
- Obesity is strongly associated with adipose tissue dysfunction and altered serum levels of adipokines, including leptin.
- Data concerning the effect of leptin on CRC development are still contradictory.

What are the new findings?

- The proliferative activity of the normal colonic epithelial cells was significantly increased in the obese model, but tumour cell proliferation was significantly lower in leptin-deficient tumour, and tumour growth was dramatically inhibited in the leptin-deficient and leptin receptor-deficient mice despite their severe obesity.
- Leptin receptor (ObR) expression levels were increased markedly in colon tumours as compared with the normal epithelium, and, in vitro, β-catenin knockdown decreased ObR expression and stimulation of recombinant Wnt increased ObR expression.
- The ability of leptin to regulate CRC growth was mediated by colonic leptin signalling via the ObR/signal transducer and activator of transcription 3 (STAT3) pathway.

How might it impact on clinical practice in the foreseeable future?

- Leptin acts as a growth factor for CRC at stages subsequent to tumour initiation in colon carcinogenesis.
- Our findings suggest that leptin signalling is a direct pathway that is crucial for CRC growth, which is a reasonable explanation for the tendency of CRC to be more aggressive in obese individuals known to show elevated serum leptin levels.
- Inhibition of leptin signalling may be efficacious for therapy and prevention of colonic adenoma and cancer with Wnt signalling activation.

INTRODUCTION

Obesity increases the risk of not only cardiovascular disease and type 2 diabetes mellitus,[1] but also of various types of cancers.[2 3] In particular, obesity has been shown to be associated with advanced progression of colorectal cancer (CRC).[4] For a number of cancers, including CRC, the risk of the disease is also elevated in individuals with obesity.[5] Epidemiological studies have revealed that obesity, especially visceral adipose tissue, raises the risk of colon adenoma[6] and CRC,[7] and the results of

animal experiments suggest a link between obesity and CRC.[7] Obesity is strongly associated with adipose tissue dysfunction and altered serum levels of adipokines, which might underlie the risk of CRC, but no definitive conclusions have been reached. Leptin, a 16-kDa product of the *ob* gene involved in energy balance and regulation of food intake,[8] is secreted predominantly in adipose tissue and is correlated with the percentage of body fat.[9] Serum leptin levels are markedly elevated in obese individuals,[10] and thus we hypothesised an association between this adipokine and increased risk of CRC.

Data concerning the effect of leptin on CRC development are contradictory and difficult to interpret.[11–20] In humans, several case–control studies have shown an elevated risk of CRC associated with high serum leptin level,[11 12] although in some studies, no elevation of the serum leptin levels were found in patients with CRC.[13 14] In experimental studies, although there has been general agreement that leptin acts as a growth factor for colon cancer cells in vitro,[15–17] conflicting results have been reported from in vivo studies that have investigated the effects of leptin on rodent colonic epithelial cell proliferation[15 18] and colon carcinogenesis.[19 20] Overall, the role of leptin in CRC induction and growth remains unclear.

Here, we explored the hypothesis that leptin signalling might regulate the growth of CRC to account for the clinical observation that obesity correlates with increased progression of CRC. We confirmed that ablation of leptin or leptin receptor (ObR) markedly inhibited the growth of colon tumours. Furthermore, we found that the ability of leptin to regulate CRC growth was mediated by colonic leptin signalling via the ObR/signal transducer and activator of transcription 3 (STAT3) pathway. This suggests that leptin signalling is a direct pathway that is crucial for CRC growth, which is a reasonable explanation for the tendency of CRC to be more aggressive in obese individuals who are known to show elevated serum leptin levels.

MATERIALS AND METHODS

Animals and tumour induction

Six-week-old male C57BL/6J-*ob/ob* mice, C57BL/KsJ-*db/db* mice, and their respective control C57BL/6J and C57BL/KsJ mice (wild-type; WT) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The animals were fed either a normal diet (ND) or high-fat diet (HFD) until the end of the study (Supplementary figure 1). The compositions of the ND (MF; Oriental Yeast Co., Tokyo, Japan) and the HFD (High Fat Diet 32; CLEA Japan Inc., Tokyo, Japan) have been described previously.[21]

The protocols for azoxymethane (AOM)-induced aberrant crypt foci (ACF) or the tumour model were essentially as described previously.[22] Briefly, mice were given 2- or 6-weekly intraperitoneal (i.p.) injections of 10 mg/kg AOM (Sigma, St. Louis, Missouri, USA) and were killed at 6 or 21 weeks following the initiation of AOM injection (Supplementary figure 1). Macroscopic tumours were counted and measured with a caliper. To facilitate the small tumour counting, the colons were stained with 0.2% methylene blue solution and were observed using stereomicroscopy. The number of ACF was counted as described previously.[22] We repeated each experiment three times to confirm the reproducibility of our results.

Leptin treatment

Ob/ob mice were divided into two groups of eight mice each, injected with either leptin or vehicle. Leptin-treated mice received daily i.p. injections of 2 μg murine recombinant leptin protein (Peprotech, Rocky Hill, New Jersey, USA) per gram of body weight for 6 weeks. Vehicle-treated mice received a 0.9% saline endotoxin-free solution for 6 weeks, which was also used for leptin injection.

Assay for proliferation and apoptosis

The entire colon was removed, gently flushed with saline to remove any faecal contents, opened longitudinally, and fixed in 10% neutralised formalin. Paraffin sections were prepared at 3 μm thickness, and stained with H&E. We evaluated the 5-bromo-2-deoxyuridine (BrdU) (BD Biosciences, Franklin Lakes, New Jersey, USA) labelling index to determine the proliferative activity of the colonic epithelial cells as described previously.[21] The apoptotic tumour cells were stained using a transferase deoxytidyl uridine end labelling (TUNEL) staining kit according to the manufacturer's instructions (Wako Pure Chemical, Osaka, Japan).

Immunohistochemistry, immunofluorescence and immunoblotting

Paraffin-embedded sections were deparaffinised and subjected to immunohistochemical staining with primary antibodies using a Histofine kit (Nichirei, Tokyo, Japan) in accordance with the manufacturer's instructions. Nuclear counterstaining was performed with haematoxylin. In the negative controls, the primary antibody was replaced by non-specific, non-immune immunoglobulin of the same isotype at an equivalent final concentration. For immunofluorescence of the cells, the cells grown on coverslips were paraformaldehyde-fixed and permeabilised with 100% ethanol at −20°C. Fixed cells were incubated with the primary antibodies and stained with Alexa Fluoro-conjugated secondary antibodies (Molecular Probes, Eugene, Oregon, USA). Nuclei were stained by 4′-diamidine-2′-phenylindole hydrochloride (DAPI; Molecular Probes). Confocal laser scanning microscopic images were then generated (Olympus, Tokyo, Japan).

Protein extracts were separated using SDS/PAGE, and the separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Amersham, London, UK). The membranes were probed with primary antibodies and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, Maryland, USA). Horseradish-peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection kit (Amersham) were used for the detection of specific proteins.

Antibodies used were anti-p-ObR, anti-ObR (Santa Cruz Biotechnology, Santa Cruz, California, USA), anti-p-STAT3, anti-STAT3, anti-cleaved caspase-3 (Cell Signaling Technology, Danvers, Massachusetts, USA), and anti-β-catenin (BD PharMingen, San Diego, California, USA).

RT-PCR analysis

Total RNA was extracted from the colonic epithelium using the RNeasy Mini Kit (Qiagen, Hilden, Germany). For real-time reverse transcription polymerase chain reaction (RT-PCR), total RNA was reverse-transcribed into cDNA and amplified using real-time quantitative PCR using the ABI PRISM 7700 System (Applied Biosystems, Foster City, California, USA). Probes and primer pairs specific for ObRb and β-actin were purchased from Applied Biosystems. The concentrations of the target genes were determined using the delta-delta Ct method and the values were normalised to those of the internal control. Primer sequences are listed in the Supplementary Methods.

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Cell culture and transfection

Colon cancer cell line SW480 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), while human embryonic kidney cells HEK 293 cells were grown in DMEM. Transfection of siRNA was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA). The cells transfected with β-catenin siRNA (Invitrogen) were harvested at 48 h after transfection, and immunoblotting and RT-PCR analysis were performed. To confirm the Wnt3a requirement for ObR expression, the cells were grown under recombinant Wnt3a protein (Stem Cell Technologies, Vancouver, British Columbia, Canada) supplementation.[23] The cells stimulated with 160 ng/ml of Wnt3a were harvested at 48 h, and RT-PCR and immunofluorescence analyses were performed.

Statistical analysis

Statistical analysis for comparisons of the number of ACF, the number and size of colon polyps, the BrdU labelling index, and the blood test results were conducted using the Mann–Whitney U test. Other statistical analyses were performed using the Student t test. Values of $p<0.05$ were regarded as denoting statistical significance.

RESULTS

Leptin regulates colorectal tumour growth, but does not stimulate the formation of ACF

To investigate the impact of leptin on obesity-related colorectal carcinogenesis and to determine whether it might act as a tumour promoter, we examined the formation of chemically induced ACF, as a marker of experimental colorectal carcinogenesis,[24] and of polyps in the colon specimens. The experimental protocol based on AOM treatment is shown in Supplementary figure 1. We used both dietary (HFD) and genetic (leptin-deficient; *ob/ob*) models of obesity for comparison with lean controls. To avoid the possibility that the differences in tumourigenecity of AOM could be due to the effects of dietary alterations on AOM metabolism, we examined the ACF model by alternating the diet 1 week after the last injection of AOM (Supplementary figure 2). The body weights and visceral fat were much higher in the *ob/ob* and WT mice fed a HFD than in the WT mice fed a ND (Supplementary figure 3). As expected, HFD exposure increased the serum leptin levels in WT mice; meanwhile, the levels of insulin and cholesterol were significantly higher in *ob/ob* mice than in WT mice, and there was no significant difference in the serum adiponectin level between the WT and *ob/ob* mice (figure 1A). We found that BrdU labelling index of the normal mucosa was significantly higher in the obese than in lean WT mice (figure 1B,C). The number of ACF in the obesity model was also significantly higher than in lean controls (figure 1D,E, Supplementary figure 3). These results suggest that obesity enhanced the development of early-stage colorectal carcinogenesis irrespective of leptin signalling.

Therefore, we focused on the later stages of cancer progression and observed that leptin deficiency dramatically decreased the tumour sizes despite *ob/ob* mice developing overt obesity (figure 2A–C,E). These findings were closely correlated with the serum levels of leptin, but were not associated with the dietary conditions. It was noteworthy that the absence of leptin had a stronger effect on colonic tumour growth than either HFD exposure or hyperinsulinaemia, which have also been reported to increase the risk of CRC.[25 26] Tumour multiplicity was also reduced more in *ob/ob* than in WT mice, but was comparable under HDF conditions (figure 2A,D). Supplementary table 1 summarises the histological findings of the tumours in WT and *ob/ob* mice.

Next, we analysed cell proliferation and apoptosis in WT and leptin-deficient tumours to explain the differences in tumour growth. We found that BrdU incorporation was significantly lower in tumours of *ob/ob* mice than WT mice (figure 2F,G), which was consistent with decreased tumour growth in the absence of leptin. Interestingly, TUNEL and cleaved caspase-3 revealed a reciprocal increase in the apoptotic response of the colon tumours between WT and *ob/ob* mice (Supplementary figure 4), which suggests that tumour cell survival also relies on leptin signalling. Taken together, these data indicate that leptin enhances tumour proliferation, but it might not exert the same effects on normal mucosa and premalignant lesions.

Leptin receptors are required for colorectal tumour growth

To clarify why the effects of leptin are limited to tumour cells, we investigated the roles of ObR in colon. We found strong ObR expression in tumour cells, but little expression in normal epithelial cells of the colonic mucosa (figure 3A,B). Expression of the long form of ObR (ObRb) mRNA was found to be significantly higher in colon tumours than in normal mucosa (figure 3C). Therefore, differences in cell proliferation dependence on leptin between tumours and normal mucosa might be explained by altered expression of ObRb.

Furthermore, to elucidate the contribution of ObRb to colonic tumourigenesis, we used mice with ObRb deletion (*db/db* mice).[27] As expected, *db/db* mice, which exhibited the same obese phenotype as the *ob/ob* mice (Supplementary figure 5), were devoid of ObRb mRNA in colonic mucosa, whereas WT mice expressed ObRb (figure 3D). In the tumour experimental protocol (Supplementary figure 1B), we observed a significant increase in the frequency and size of tumours in WT mice as compared to *db/db* mice (figure 3E–G); meanwhile, there was no significant difference in tumour size and multiplicity between the *db/db* mice fed ND and those fed a HFD (Supplementary figure 6). Supplementary table 2 summarises histological findings of tumours in WT and *db/db* mice. On the other hand, the number of ACF in *db/db* mice was significantly higher than in WT mice (Supplementary figure 7). These results suggest that epithelial ObRb is required for transduction of tumour-promoting signals from leptin.

Wnt signalling stimulates expression of ObRb

We explored the mechanism of ObRb expression in tumours. Expression of ObRb was strong in the tumour epithelium where Wnt signalling was activated. Frequent gene mutations of β-catenin and altered cellular localisation of the protein are features of AOM-induced colon tumours in mice.[28 29] Using immunohistochemical analysis, we examined the expression of β-catenin in colon tumours induced by AOM in comparison with that in the adjacent normal mucosa. Cytoplasmic and nuclear β-catenin staining was pronounced in all tumour tissues of WT and *ob/ob* mice, whereas antibody binding was limited to the membranes at the intercellular borders in normal epithelial cells (Supplementary figure 8). Importantly, the stabilised β-catenin in the nuclei was observed in tumour, irrespective of leptin deficiency. To elucidate the roles of Wnt signalling activation in the regulation of leptin/ObRb signalling, we examined the effects of β-catenin knockdown on leptin/ObRb pathway in the human SW480 colon cancer cell line. Transfection of siRNA for the β-catenin gene markedly reduced the protein expression level (figure 4A). Notably, Wnt signalling inhibition by β-catenin

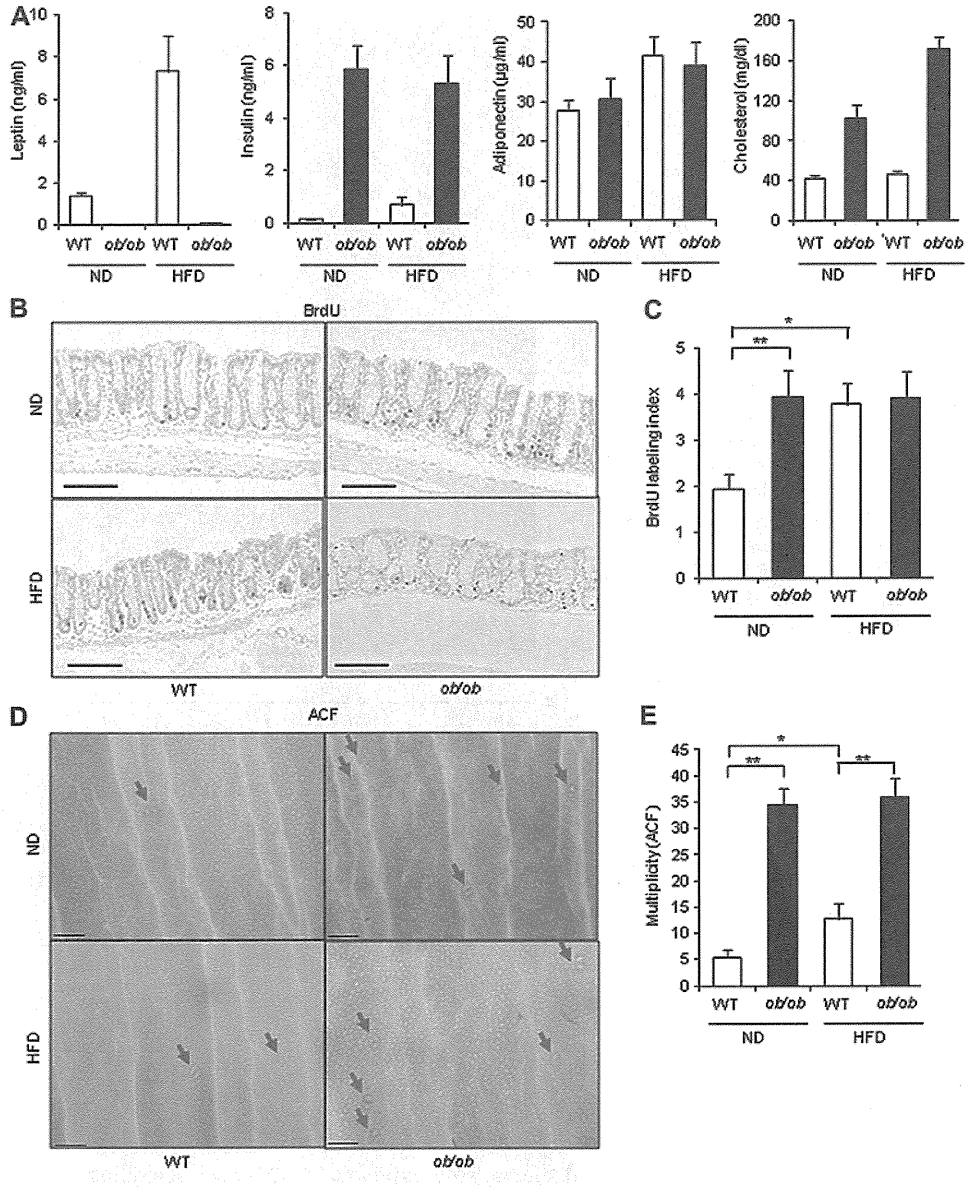

Figure 1 Leptin is not involved in the early-stage of colorectal carcinogenesis in obesity. (A) AOM-treated wild-type (WT) and *ob/ob* mice were bled and the levels of metabolic factors in serum were determined by ELISA. Results are averages ± SEM. (n=8). ND, normal diet; HFD, high-fat diet. (B) BrdU incorporation in normal colonic epithelial crypts of WT and *ob/ob* mice. Scale bars=100 μm. (C) BrdU labelling indices of colonic normal mucosa of AOM-treated WT and *ob/ob* mice. Results are averages ± SEM. (n>10). *p<0.01. **p<0.005. (D) Stereoscopic observations of ACF (arrows) in colonic tissues of WT and *ob/ob* mice. The samples were stained with 0.2% methylene blue. Scale bars=100 μm. (E) ACF multiplicity. Results are averages ±SEM. (n=10). *p<0.05. **p<0.001. ACF, aberrant crypt foci; AOM, azoxymethane; BrdU, bromodeoxyuridine; WT, wild type.

knockdown markedly reduced ObRb expression (figure 4A). We confirmed that the ObRb mRNA level was significantly reduced in the β-catenin siRNA-transfected SW480 cells (figure 4B). Reductions of ObRb protein expression by transfection of β-catenin siRNA was also observed in other colon cancer cell lines (Supplementary figure 9). It has been shown that the Wnt/β-catenin pathway can be stimulated in HEK293 cells by addition of Wnt3a.[30] We tested ObRb expression level following Wnt3a stimulation in HEK293 cells that normally contain trace amounts of nuclear β-catenin. Wnt3a-stimulated HEK293 cells showed a marked increase in ObR expression (figure 4C,D). These results are consistent with the increased expression levels of ObR in tumours as compared with those in normal mucosa (figure 3A–C), and indicate that the Wnt signalling activates ObRb expression in colonic epithelium.

Leptin activates STAT3 signalling to promote colorectal tumour growth

Phosphorylation of Tyr^{1138} in ObRb induces STAT3 activation.[31] Increased amounts of phosphorylated Tyr^{1138}-ObRb and STAT3 (p-STAT3) were observed in tumours as compared with those in normal mucosa (figure 5A). These data suggest that leptin exerts a stimulatory action on colon tumours through the ObRb/STAT3 pathway.

To determine the contribution of leptin to changes in tumour cell proliferation and survival, we analysed colon tumours of WT and *ob/ob* mice for activation of STAT3 and the expression of its target genes. Immunohistochemical analysis revealed tumour cell nuclear localisation of p-STAT3 in colon tumour cells in WT mice, while this signal was almost completely absent from similar tumours in *ob/ob* mice (figure 5C). The frequency of p-STAT3-positive cells was significantly higher in tumours of WT mice fed a HFD than in those of the mice fed a ND (figure 5C, Supplementary figure 10), closely matching the increase in serum leptin levels (figure 1A). Meanwhile, p-STAT3-positive cells were almost undetectable in normal mucosa of WT and *ob/ob* mice (figure 5B). Importantly, the lack of STAT3 activation in colonic mucosa coincided with the lack of colonic leptin signalling, namely, lack of functional leptin (figure 1A) or lack of colonic ObR expression (figure 3A–C). Therefore, our data indicate that leptin is a crucial STAT3 activator in colonic epithelium during tumour growth. Next, we analysed the STAT3-mediated proliferative response in WT and leptin-deficient tumours. To do that, we investigated the expression of

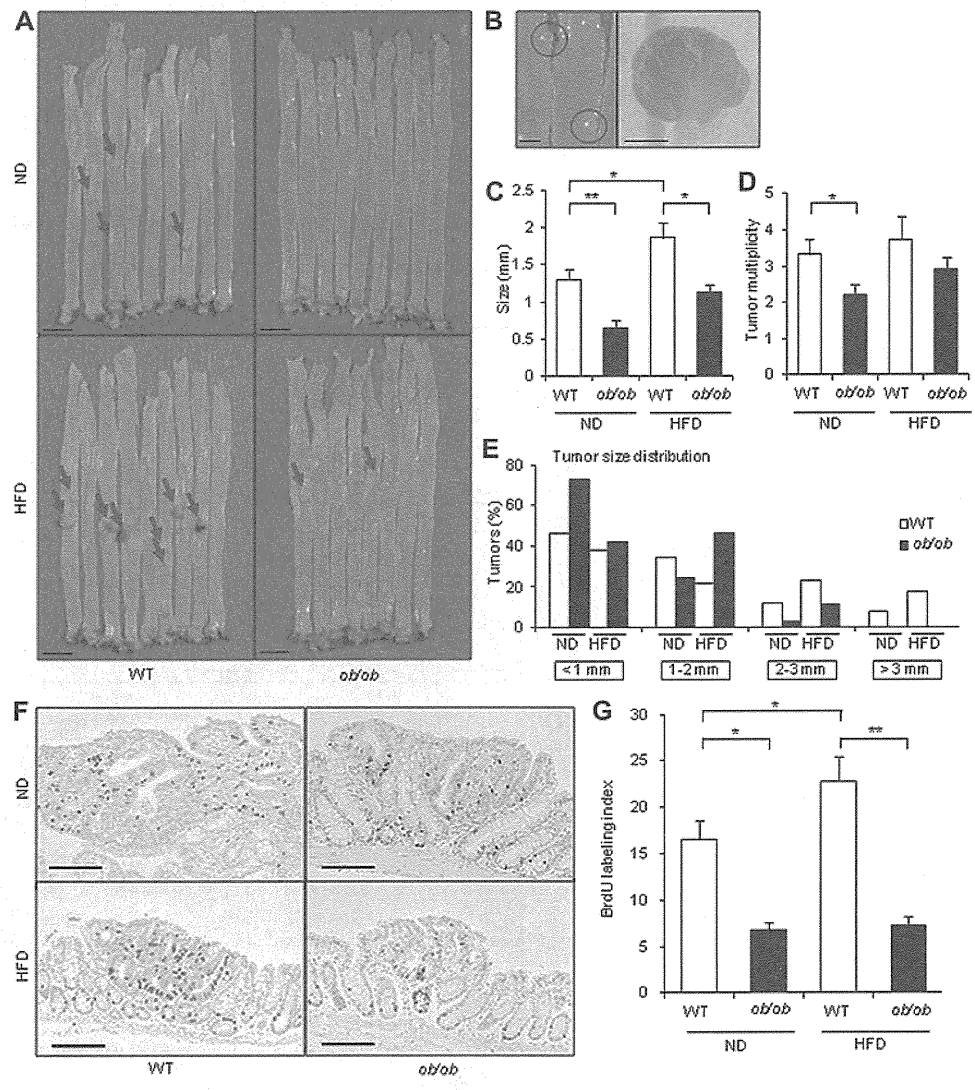

Figure 2 Leptin regulates AOM-induced colon tumour growth. (A) Macroscopic findings of colon tumours. Arrows indicate large tumours. Scale bars=1 cm. ND, normal diet; HFD, high-fat diet. (B) Macroscopic (left panel) and stereomicroscopic (right panel) findings of small colon tumours. Scale bars=1 mm (left panel) and 200 μm (right panel). (C) Tumour size. Results are averages ±SEM. (n=10). *p<0.05. **p<0.005. (D) Tumour multiplicity in WT and *ob/ob* mice fed ND or HFD. Results are averages ±SEM. (n=10). *p<0.05. (E) Histogram showing size distribution of tumours. (F) BrdU incorporation in colon tumour of WT and *ob/ob* mice. Scale bars=100 μm. (G) BrdU labelling indices in colon tumours of the AOM-treated WT and *ob/ob* mice. Results are averages ±SEM. (n>10). *p<0.05. **p<0.005. AOM, azoxymethane; BrdU, bromodeoxyuridine; WT, wild type.

cell-cycle genes and of the cyclin-dependent kinase (Cdk) inhibitor $p21^{cip}$ in tumours. We found that mRNA expressions of cyclin D1, c-Myc, cyclin B1, cyclin E and cdc2 were increased to a greater degree in WT mice than in *ob/ob* mice (figure 5D). This suggested a stimulatory effect of STAT3 on the cell cycle, and this observation was consistent with the downregulation of the Cdk inhibitor $p21^{cip}$ in tumours of WT mice. Furthermore, we observed elevated expression levels of Bcl-X_L and survivin in WT mice as compared with those in *ob/ob* mice (figure 5D). These results suggest that impaired induction of Bcl-X_L and survivin protein expression may account for the increased rate of apoptosis observed in leptin-deficient *ob/ob* mice. Collectively, these results strongly support the notion that the STAT3-associated proliferative and antiapoptotic effects are important for tumour epithelia.

Exogenous leptin compensates for suppressed tumour growth in leptin-deficient mice

We found that continuous treatment with recombinant leptin during the late-stage of CRC (figure 6A) resulted in an increase of tumour sizes (figure 6B–D), whereas tumour multiplicity was not affected (figure 6E). Supplementary table 3 summarises the histological findings of tumours. As expected, treatment with recombinant leptin resulted in elevated serum levels of leptin (figure 6F). Importantly, leptin supplementation enhanced STAT3 phosphorylation in colonic tumours (figure 6G). Thus, leptin signalling can increase tumour size without affecting tumour multiplicity, which has an impact on tumour growth.

DISCUSSION

The existence of a relationship between obesity-related factors and CRC has been speculated upon in recent years, but no definitive conclusions have been reached. The present investigation to elucidate the precise mechanisms involved was necessary because of the major clinical implications. We identified a novel mechanism to explain how leptin deficiency might suppress colon tumour growth, even in the presence of marked increase in the levels of other obesity-related factors. Our finding suggests that leptin is a crucial factor for colon tumour growth among the various obesity-related factors. We demonstrated an increase in the proliferative activity of the normal colonic epithelial cells and ACF formation in the obesity model but, unexpectedly, tumour growth was inhibited dramatically in the leptin-deficient obesity model, indicating the importance of leptin signalling for colon tumour growth. Taken in combination, our data indicate that leptin acts as growth factor for CRC at stages subsequent to cancer initiation.

Previous studies have provided much evidence of an association between metabolic factors and increased risk of colorectal carcinogenesis.[7] Therefore, we hypothesised at first that *ob/ob*

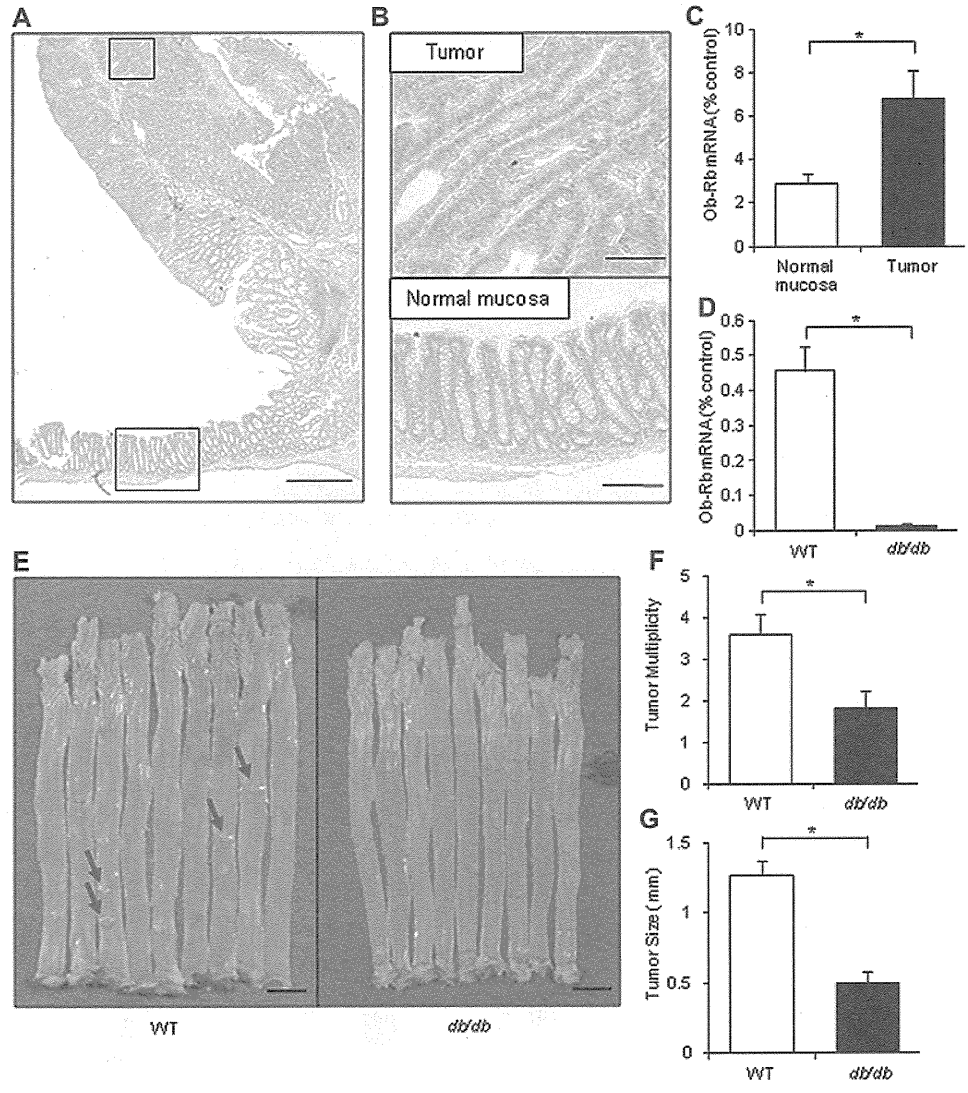

Figure 3 Leptin receptors are required for colorectal tumour growth. (A) and (B) Section of colon from an AOM-treated mouse showing a representative tumour protruding into the colonic lumen was stained with antibodies to ObR. Magnified views of the boxed area confirms the presence of ObR-positive cells in the cytoplasm of tumour cells (upper panel), but scarce expression of ObR in normal epithelial cells (lower panel) of the colonic mucosa. Scale bars=400 μm (left panel), 10 μm (right, upper panel), and 100 μm (right, lower panel). (C) Relative expression level of ObRb mRNA in normal colonic mucosa and tumours of AOM-treated mice, analysed by real-time PCR. Results are averages ±SEM. (n=5). *$p<0.005$. (D) Expression of ObRb in the isolated colonic mucosa from WT and leptin-receptor-deficient *db/db* mice. Results are averages ±SEM. (n=5). *$p<0.005$. (E) Macroscopic findings of the colon tumours. Arrows indicate large tumours. Scale bars=1 cm. (F) Tumour multiplicity in WT and *db/db* mice subjected to induction of colon tumours. Results are averages ±SEM. (n=10). *$p<0.05$. (G) Tumour size in WT and *db/db* mice. Results are averages ±SEM. (n=10). *$p<0.0001$. AOM, azoxymethane; WT, wild type.

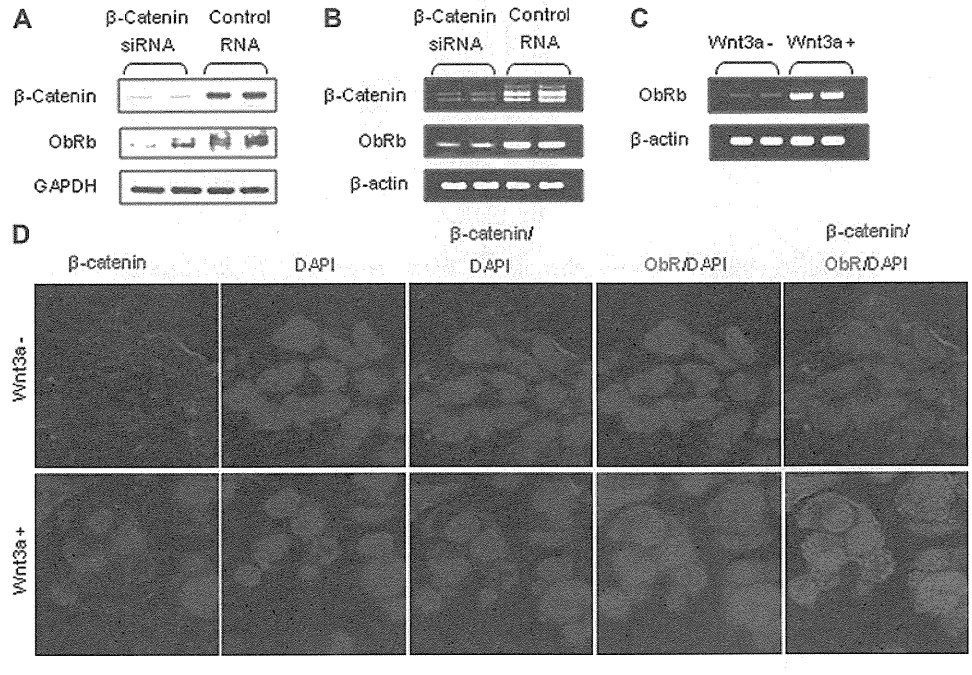

Figure 4 Wnt signalling increases the expression levels of ObRb. Western blot (A) and RT-PCR (B) analyses of ObRb expression in SW480 cells transfected with β-catenin siRNA (50 nM). Samples were prepared 48 h after transfection. ObRb mRNA and protein expression was decreased by β-catenin siRNA. GAPDH and β-actin are shown as the loading controls. (C) RT-PCR analysis of ObRb expression in HEK293 cells with and without Wnt3a stimulation. ObRb mRNA expression levels were increased by Wnt3a stimulation. β-actin is shown as a loading control. (D) Immunofluorescence of β-catenin (red) and ObR (green) in HEK293 cells treated with recombinant Wnt3a. Wnt3a induced translocation of β-catenin from the cytoplasm to the nuclei and induced the expression of ObR. DAPI, 4′-diamidine-2′-phenylindole hydrochloride; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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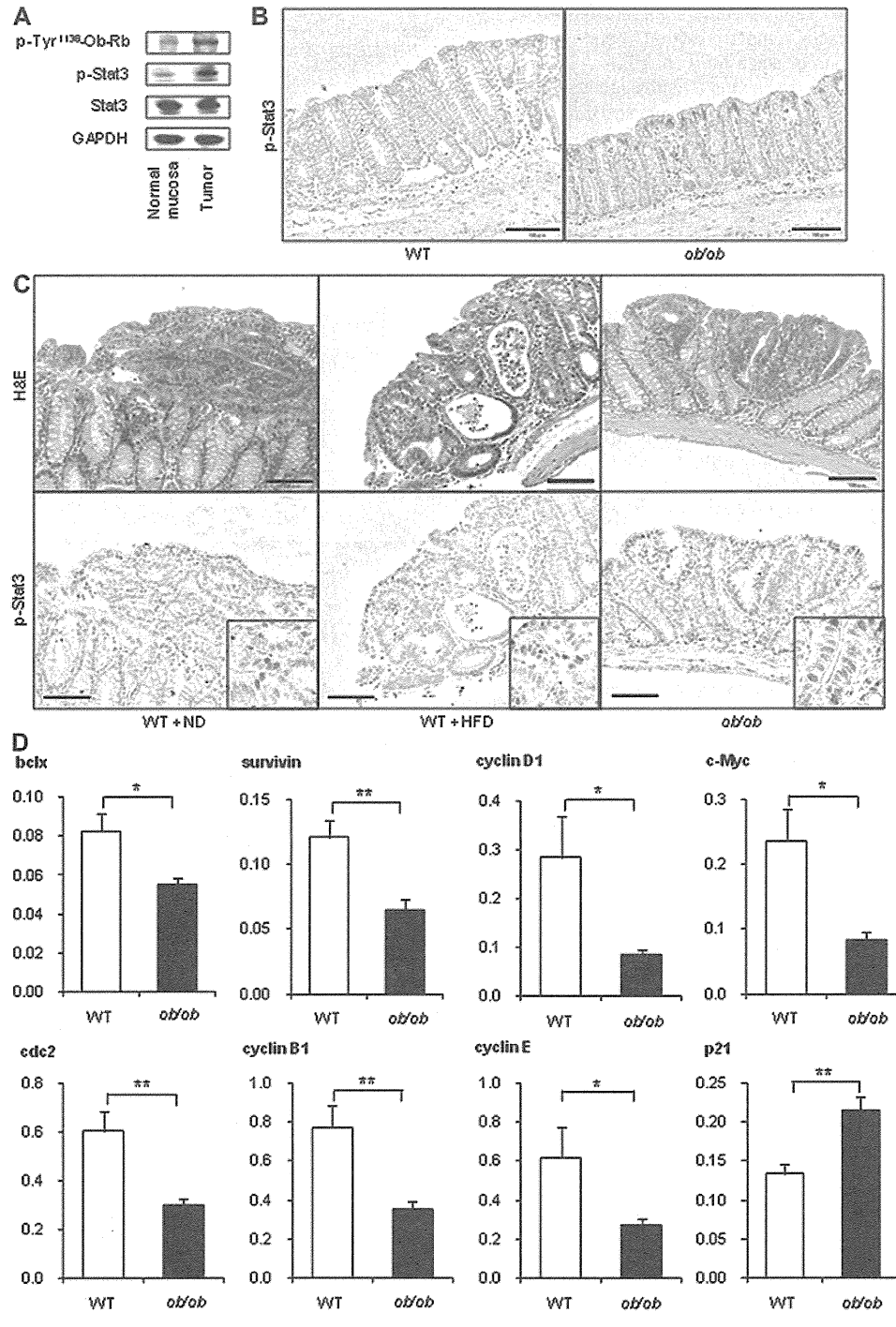

Figure 5 Leptin increases STAT3 phosphorylation in colon tumours. (A) Immunoblot analysis for Tyr^{1138}-phosphorylated ObRb and phosphorylated STAT3 (p-STAT3) in normal colonic mucosa and tumours of AOM-treated mice. (B) Paraffin-embedded sections of tumour-containing colons from WT and *ob/ob* mice were stained with H&E (upper panel) and with anti-p-STAT3 (lower panel). Insets in lower panels demonstrate nuclear localisation of p-STAT3 in WT mice, which was absent from tumours in *ob/ob* mice. Scale bars=100 μm. ND, normal diet; HFD, high-fat diet. (C) Normal colon mucosa from AOM-treated WT and *ob/ob* mice was stained with anti-p-STAT3. Scale bars=100 μm. (D) Expression of cell-cycle and apoptosis regulators in isolated colonic tumours from WT and *ob/ob* mice. Relative mRNA expression levels were determined by real-time PCR. Results are averages ±SEM. (n=6). *p<0.05. **p<0.01. AOM, azoxymethane; WT, wild type.

mice, which have obese metabolic phenotypes with elevated levels of insulin, glucose and lipid, would show increased susceptibility to CRC development as compared to their lean littermates. However, to our surprise, we found that *ob/ob* mice developed far smaller tumours than the corresponding WT mice, despite the animals exhibiting severe obesity. In contrast, administration of a HFD to WT mice resulted in increased tumour sizes, despite the finding that levels of various obesity-related metabolic factors, with the exception of leptin, in these mice were not as high as those in *ob/ob* mice. These results strongly indicate that, in vivo, leptin is important for the regulation of colon tumour growth, irrespective of obesity. Furthermore, these results also explain that CRC does not grow under leptin-deficient conditions, regardless of the serum insulin levels. Leptin-deficient mice exhibited few and small tumours despite a high intake of dietary fat. These findings suggest that leptin is a crucial factor for CRC development, regardless of dietary composition. Adiponectin has also been reported to influence colorectal carcinogenesis. Recently, we have demonstrated that adiponectin deficiency might promote the development of CRC only under HFD conditions, using adiponectin-knockout mice.[21] Furthermore, a human epidemiological study has shown that decreased levels of plasma adiponectin are associated with increased risk of CRC.[32] In a cell model study, adiponectin has been shown to block leptin-induced colon epithelial cell proliferation.[33] However, there was no significant difference in the serum adiponectin level between the WT and *ob/ob* mice in the present study. We speculate that there are

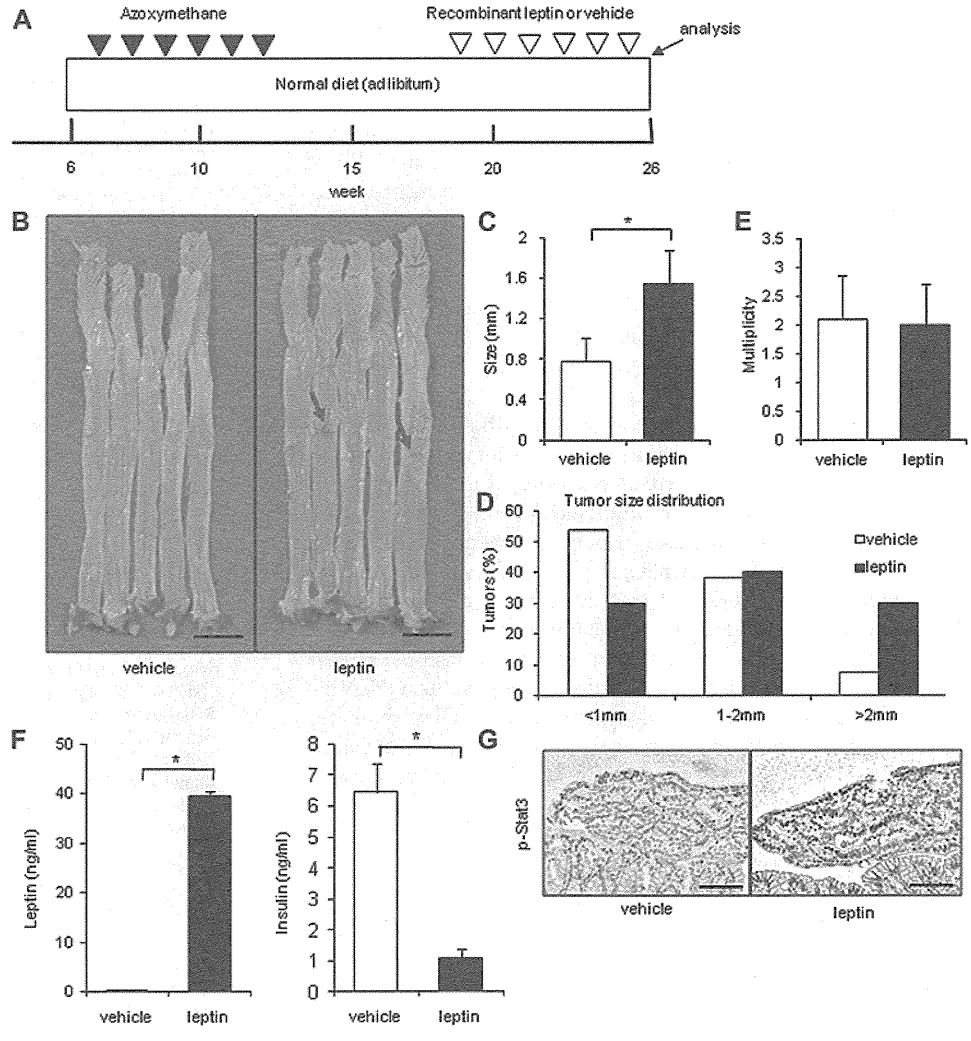

Figure 6 Leptin signalling stimulates tumour growth. (A) Scheme of treatment with recombinant leptin during the late stage of CRC growth. Mice were injected i.p. with 2 μg recombinant leptin, or control saline every day from 15 weeks after initial AOM injection. (B) Macroscopic findings of colon tumours. Arrows indicate large tumours. Scale bars=1 cm. (C) Tumour size. Results are averages ±SEM. (n=6). *p<0.05. (D) Histogram showing size distribution of colon tumours. (E) Tumour multiplicity. Results are averages ±SEM. (n=6). (F) Vehicle- or leptin-treated *ob/ob* mice were bled and the levels of leptin (left panel) and insulin (right panel) in serum were determined by ELISA. Results are averages ±SEM. (n=6). *p<0.001. (G) Paraffin-embedded sections of tumour-containing colons of vehicle- and leptin-treated *ob/ob* mice stained with anti-p-STAT3. Scale bars=100 μm. AOM, azoxymethane; CRC, colorectal cancer.

many factors that influence colon carcinogenesis in an obesity background, and adiponectin may be one of these factors. Further studies in animal CRC models are necessary to address the interaction between adiponectin and leptin.

Using genetic models, we demonstrated that leptin is an important regulator of CRC development. However, leptin signalling did not have a significant effect on promotion of premalignant lesions in the CRC model, because its absence did not alter the number of ACF. These data indicate that leptin does not act as a growth-promoting agent at an early stage of colon carcinogenesis. We observed that the number of ACF was significantly greater in *ob/ob* and *db/db* mice than in WT mice, which suggests that metabolic factors other than leptin act as promoters of early-stage colon carcinogenesis. Furthermore, leptin signalling did not promote normal colonic epithelial cell proliferation either. Why did leptin enhance tumour cell proliferation, but not induce formation of ACF or proliferation of normal colonic mucosa? Here, we noted a difference in ObR between tumours and normal mucosa. A marked increase in ObR expression level was observed in tumours as compared with that in the normal mucosa. Carcinogen-induced tumours frequently show mutation of β-catenin that leads to stabilisation and nuclear translocation of β-catenin, thereby activating the Wnt pathway. On the other hand, mutation and altered cellular localisation of β-catenin are not observed in normal mucosa or ACF.[34] Based on this evidence, we hypothesised that activation of the Wnt pathway not only triggers the formation of colon tumours, but also induces the expression of ObR in colon tumours. To elucidate the roles of Wnt signalling activation on regulation of ObR expression, we examined the effects of β-catenin knockdown on ObR expression in colon cancer cell lines, and confirmed decrease in ObR mRNA and protein levels. Furthermore, we also confirmed increased ObR expression levels in exogenous Wnt-stimulated HEK293 cells. Thus, we propose that Wnt signalling contributes to the upregulation of ObR in colonic epithelium. Based on these results, we conclude that leptin stimulates the proliferation of tumour cells that carry activating alterations in the canonical Wnt pathway (Supplementary figure 11). Furthermore, these data also suggest that leptin is not involved in early-stage colorectal carcinogenesis. Collectively, our observations provided a novel finding that leptin acts as growth factor for CRC only after the tumour initiation stage during the process of colorectal carcinogenesis (Supplementary figure 12).

Our data define a novel role for leptin signalling in the control of tumour growth in addition to its essential role in food intake and energy regulation. The role of leptin signalling is evident from the finding of increased ObR expression in colon tumours, and of such increased expression coinciding with the activation of STAT3. Furthermore, absence of leptin signalling prevented tumour growth, and suppressed STAT3 activation in these tumours. These findings demonstrated that activation of STAT3 in tumours is crucially dependent on leptin signal transduction. Finally, the leptin signalling mechanism of action was revealed

operationally by the finding that treating mice with recombinant leptin increased tumour growth. Taken together, these data provide strong evidence to indicate that leptin signalling controls tumour growth in vivo.

It has been shown previously that recombinant leptin does not stimulate cell proliferation and carcinogenesis in vivo.[16 18 20] While continuous treatment with recombinant leptin enhanced tumour growth in AOM-treated mice, the effect of exogenous leptin was not as strong as we had expected. On the other hand, there is general agreement that leptin acts as a growth factor for colon cancer cells in vitro.[15–17] These discrepancies between in vivo and in vitro studies could be explained by the complicated interaction between various hormones and cytokines. The effects of leptin in vivo are not as simple as those in vitro. Leptin is known to regulate the secretion of several hormones. Importantly, the actions of leptin involve amelioration of hyperinsulinaemia.[35 36] We observed such actions of leptin on insulin levels in mice treated with recombinant leptin. Insulin has the effect of promoting the development of chemically induced tumours in the colon.[37] Therefore, in vivo, the effects of exogenous leptin on promotion of colonic tumourigenesis might be suppressed through a decrease in insulinaemia.

In conclusion, we clearly demonstrated a relationship between leptin signalling and growth of colon tumours, using leptin-deficient or leptin-receptor-deficient mice. The dramatic suppression of colon tumour growth resulting from inhibition of leptin signalling indicates that leptin is an important growth factor for colon cancer progression. We speculate that dietary intake of excessive fat and calories might result in energy storage in the visceral and subcutaneous adipose tissue compartments, and that any surplus energy might be used for growth of CRC through leptin signalling. On the basis of the current results, it is reasonable to conceive that colon tumours might have a tendency to develop in obese individuals who over-eat and who show elevated serum leptin levels. Future study is warranted to address the importance of leptin signalling in the metastatic spread of CRC. Our data provide novel insights into leptin signalling in CRC and suggest novel therapeutic and preventive targets against colon polyps and cancers based on inhibition of leptin-dependent STAT3 signalling.

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Competing interests None.

Ethics approval All animal experiments were conducted with the approval of the institutional Animal Care and Use Committee of Yokohama City University School of Medicine.

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