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Research Article

Molecular Cancer Therapeutics

Ligand-Specific Antibodies to Insulin-Like Growth Factors Suppress Intestinal Polyp Formation in *Apc*$^{+/-}$ Mice

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Abstract

Insulin-like growth factors (IGF-I and IGF-II) play important roles in intestinal tumorigenesis. To investigate the effectiveness of IGF-targeting strategies, we conducted an *in vivo* study using anti-mouse neutralizing antibodies IGF-I (KM3168) and IGF-II (KM1468). Six- and 10-week-old *Apc*$^{+/-}$ mice were given KM3168 and/or KM1468 i.p. at two doses (0.01 or 0.1 μg/g weight) once or twice weekly for 4 weeks. To clarify the source of IGFs *in vivo*, we evaluated the expression levels of IGFs in the liver, normal small intestine, and polyps of the small intestine of *Apc*$^{+/-}$ mice. The phosphorylation status of IGF signal–related molecules was examined using immunostaining to understand the mechanism underlying the effects of IGF-neutralizing antibody. The plasma half-life was 168 for KM3168 and 85 hours for KM1468. In two lineages of *Apc*$^{+/-}$ mice (*Apc*^{1309} and *Apc*$^{Min/+}$), a low dose (0.01 μg/g weight) of KM3168 and KM1468 significantly reduced the number of polyps when given once and twice weekly, respectively. Combined administration of the effective dose of each antibody had an additive effect. The liver was the main source of IGF-I, whereas the polyps of the small intestine and normal small intestine were the main source of IGF-II. IGF-neutralizing antibodies decreased the phosphorylation of IGF type 1 receptor and inhibited the signal transduction of the Akt pathway. These results suggest that IGF-I and IGF-II play important roles in polyp formation in *Apc*$^{+/-}$ mice and that specific antibodies to IGF-I and IGF-II may be promising antitumor agents. *Mol Cancer Ther; 9(2); 419–28. ©2010 AACR.*

Introduction

Colorectal cancer is the third most common cancer and the fourth most frequent cause of cancer-related death worldwide. The possible role in promoting oncogenic transformation, growth, and survival of colorectal neoplasm of the insulin-like growth factor (IGF) system, which includes IGF-I, IGF-II, multiple IGF-binding proteins (IGFBP), and IGF type-1 receptors (IGF-IR), has been investigated (1–5). IGF-IR is a receptor common to both IGF-I and IGF-II, and binding of the receptor to these IGFs causes activation of downstream signaling cascades resulting in proliferative and antiapoptotic effects. A meta-analysis showed an association between high circulating IGF-I and IGF-II concentrations and increased risk of colorectal cancer (6, 7). Acromegalic patients whose hypersecretion of growth hormone is accompanied by elevated IGF-I levels have an elevated incidence of colorectal adenomas and cancer (8, 9). These epidemiologic data are supported by an *in vivo* study in the mouse showing genetically deleted IGF-I expression in the liver, which reduced the circulating IGF-I concentration by 75% and delayed the growth of an orthotopic xenograft. This effect was cancelled by exogenous IGF-I replacement (10).

IGF-II also plays an important role in the development of colon cancer. The IGF-II gene is an imprinting gene whose parental allele is expressed and maternal allele is silenced. Loss of imprinting of the IGF-II gene increases locally produced IGF-II concentration, which correlates with colorectal carcinogenesis (11–15). Some studies have shown that IGF-II modifies intestinal tumor growth in *Apc*$^{Min/+}$ mice, a close genotypic and phenotypic model of human familial adenomatous polyposis, with genetically altered IGF-II expression (16–18).

Recent *in vivo* studies have shown the therapeutic potential of interfering with the signaling mediated by the IGF system in cancer cells. In particular, IGF-IR is the most attractive target, and many IGF-IR–targeting strategies, including monoclonal antibodies and small-molecule tyrosine kinase inhibitors, have been developed (19–21). In contrast, few reports have been published on IGF-ligand–targeting strategies (22). We previously developed an IGF-neutralizing antibody, KM1468, and reported its therapeutic efficacy in several mouse models (23 –26). Notably, KM1468 neutralizes mouse IGF-II but does not cross-react with mouse IGF-I (23). To clarify the

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role of IGFs in tumorigenesis, the development of a specific antibody to IGF-I is essential.

In the present study, we developed and characterized a new anti–IGF-I antibody, KM3168, *in vitro*. We performed *in vivo* studies to clarify the roles of IGF-I and IGF-II in intestinal tumorigenesis and evaluated the therapeutic efficacy of IGF-neutralizing antibodies against polyp formation in two lineages of $Apc^{+/-}$ mice (Apc^{1309} and $Apc^{Min/+}$).

Materials and Methods

Animals

C57BL/6J$^{Apc/Apc\Delta 1309}$ mice (Apc^{1309} mice) were kindly provided by Tetsuo Noda, PhD (Cell Biology Department, Japanese Foundation for Cancer Research, Cancer Institute, Japan). C57BL/6-$Apc^{Min/+}$ mice ($Apc^{Min/+}$ mice) were purchased from The Jackson Laboratory. Apc^{1309} and $Apc^{Min/+}$ mice offspring were genotyped according to the method previously described (27, 28) and recommendations of The Jackson Laboratory, respectively. Male mice were randomized into experimental and control groups and were fed standard laboratory chow and tap water *ad libitum*. All mice were housed in specific pathogen-free conditions in the animal facility of Kyoto University. The studies were approved by the animal protection committee of our institution.

Cell Lines and Cell Culture

HT29 cells were purchased from the American Type Culture Collection. BALB/c 3T3 fibroblasts overexpressing human IGF-IR (3T3-IGF-IR) were kindly provided by Drs. Axel Ullrich (Max Planck Institute of Biochemistry, Department of Molecular Biology, Martinsried, Germany) and Reiner Lammers (Internal Medicine IV, Division of Diabetes Research, University of Tubingen, Tubingen, Germany). HT29 cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum, and 3T3-IGF-IR cells were propagated in DMEM (Life Technologies Laboratories) with 10% fetal bovine serum. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

IGF-Neutralizing Antibodies, KM1468, and KM3168

Monoclonal antibodies KM 1468 (rat IgG2b) and KM3168 (rat IgG2a) were generated by Kyowa Hakko Kogyo, Co. Ltd. KM1468 was selected for its ability to neutralize human IGF-I, human IGF-II, and mouse IGF-II. This antibody shows no cross-reactivity with mouse IGF-I or insulin. The detailed characterization of KM1468 has been published elsewhere (23). KM3168 is a novel monoclonal antibody against purified full-length mouse IGF-I and was established by immunizing female SD rats (SLC) with methylated bovine serum albumin conjugated to recombinant mouse IGF-I (R&D Systems). KM3168 was purified from ascites fluid by caprylic acid precipitation and ammonium sulfate precipitation as previously described (23).

Characterization of KM3168

Proliferation Inhibition Assay. To measure the neutralizing activity of KM3168, HT-29 cells were starved in serum-free medium (DMEM/F12, phenol red free; Invitrogen) supplemented with 10 μg/mL human transferrin (Life Technologies Laboratories) and 200 μg/mL bovine serum albumin fraction V (Invitrogen) for 24 h. Cells were harvested; 5×10^3 cells were seeded in a 96-well tissue culture plate; and various concentrations of mouse IGF-I (40 ng/mL), mouse IGF-II (40 ng/mL), human IGF-I (10 ng/mL), human IGF-II (10 ng/mL; R&D Systems), or insulin (40 ng/mL; Wako) were added with various concentrations of KM3168 (up to 10 μg/mL). After 5 d of incubation, viable cells were detected by addition of the proliferation reagent WST-1 (Roche), and the cells were incubated at 37°C for a further 1 to 3 h. After incubation, the plates were shaken thoroughly and the color was quantified by measuring the absorption at 450 nm on an E_{max} microplate reader (Molecular Devices). The data are expressed as the mean obtained from triplicate experiments.

IGF-IR Phosphorylation Inhibition Assay. BALB/c 3T3 IGF-IR cells at 70% confluency were starved in serum-free medium for 24 h and then incubated with 10 ng/mL of mouse IGF-I, mouse IGF-II, human IGF-I, human IGF-II, or insulin with various concentrations of KM3168 (up to 10 μg/mL) for 30 min. Two hundred microliters of each 3T3- IGF-I R cell lysate were prepared as previously described (29). The cell lysates (10 μg) were separated by 7.5% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, probed overnight at 4°C with an anti–phospho-IGF-IR rabbit polyclonal antibody (BioSource), and visualized by enhanced chemiluminescence (Amersham). Phosphorylation of IGF-IR was detected using an LAS-3000mini image reader (Fujifilm) and quantified using Multi-Gauge software (Fujifilm). The 50% neutralizing dose (ND_{50}) of KM3168, defined as the dose that blocked 50% of the phosphorylation of the IGF-IR induced by various IGFs relative to that in KM3168-nontreated cells, was calculated using the GraphPad Prism software (GraphPad Software). The inhibition ratio is expressed as a percentage and was calculated as (1 – absorbance of KM3168-treated cells/absorbance of KM3168-nontreated cells) × 100.

***Pharmacokinetics of KM3168* In vivo.** KM3168 (200 μg/mouse) was injected i.p. into three 6-wk-old C57BL/6J mice, and blood samples were taken from the retro-orbital venous plexus of these mice at five times: before injection, and 1, 2, 4, and 7 d after injection. Samples were centrifuged at 1,000 × *g* for 5 min, and the KM3168 (rat IgG) concentration was measured in plasma samples using a rat IgG ELISA kit (Bethyl Laboratories).

Effects of IGF-Neutralizing Antibodies on Intestinal Polyp Formation

The IGF-I–neutralizing antibody KM3168, the IGF-II–neutralizing antibody KM1468, and the control rat

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IgG (Chemicon) solutions were prepared in sterile PBS and stored at −80°C until use. These substances were injected i.p. into Apc^{1309} mice and $Apc^{Min/+}$ mice according to the following two experimental designs. To estimate the major systemic adverse event of IGF-neutralizing treatment, body weight (weekly), and blood glucose level (before and after treatment) were monitored.

Experiment 1. This study was designed to determine the optimal dose of each antibody for treatment of intestinal polyp formation in Apc^{1309} mice. KM3168 or KM1468 was injected i.p. into Apc^{1309} mice at a dose of 0.01 or 0.1 μg/g weight once or twice weekly for 4 wk from 6 to 10 wk of age. Rat IgG was injected into littermates as the negative control using the same method.

Experiment 2. This study was designed to clarify the combined effect of KM3168 and KM1468. Based on the results of experiment 1, KM3168 alone (0.01 μg/g weight weekly), KM1468 alone (0.01 μg/g weight twice weekly), or both antibodies together were injected i.p. into Apc^{1309} mice and $Apc^{Min/+}$ mice for 4 wk from 6 to 10 wk of age (early intervention) and from 10 to 14 wk of age (late intervention). Rat IgG was injected as the negative control using the same method.

Assessment of Intestinal Polyps

All animals were killed humanely by cervical dislocation after the completion of treatment, and the intestinal tracts were removed. After flushing with PBS, the small intestine was opened longitudinally, cleaned, and fixed flat between sheets of filter paper in 7% formalin. Tumors were investigated under a dissecting microscope at ×20 magnification by two investigators blinded to the treatment of each mouse. The number, diameter, and distribution of tumors were obtained for the entire length of intestine. The percentage of polyp area (%polyp area) in the small intestine was calculated as the ratio of the sum of every polyp area (tumor radius^2 × π, in mm^2) to the total small intestine area (multiples of length of each fixed segment by width at the midpoint of each segment).

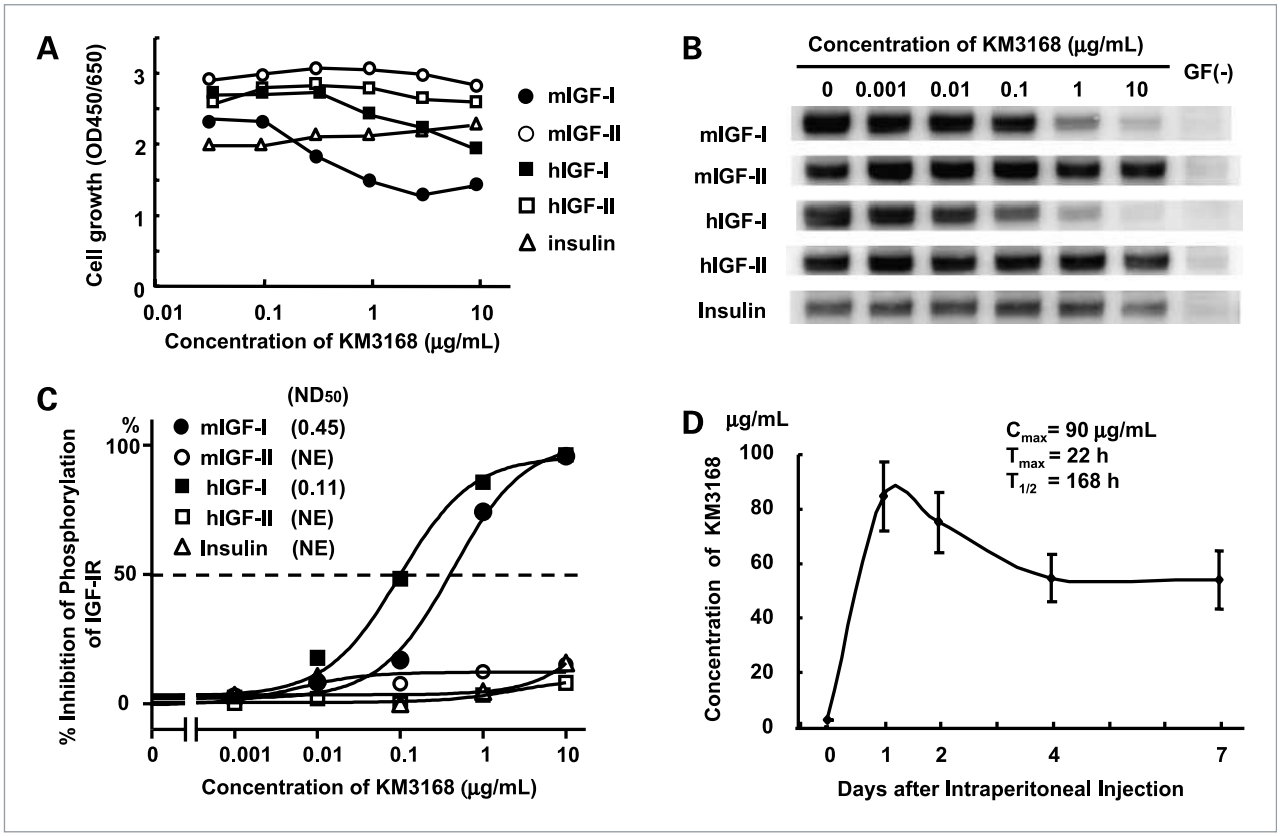

Figure 1. Characterization of KM3168. A, determination of the effects of KM3168 neutralizing activity on various IGF factors in HT29 cells after stimulation by the proliferation reagent WST-1. KM3168 neutralized human IGF-I (hIGF-I) and mouse IGF-I (mIGF-I) but did not neutralize hIGF-II, mIGF-II, or insulin. Points, mean (n = 3 per assay). B, KM3168 inhibited the phosphorylation of human and mouse IGF-I–stimulated IGF-IR but did not inhibit the phosphorylation of human or mouse IGF-II–stimulated or insulin-stimulated IGF-IR. The phosphorylation status of IGF-IR was evaluated by the Western blotting analysis using an anti–phospho-IGF-IR antibody. GF (–), nontreated cells. C, the bands on the Western blotting shown in B were scanned and quantified with a densitometer. The inhibition ratio (expressed as a percentage) was calculated as: (1 – absorbance of KM3168-treated cells/absorbance of nontreated cells) × 100. NE, not evaluated. D, profile of plasma KM3168 antibody levels after a single i.p. injection in wild-type mice (200 μg/mouse). Mice were tested five times using three mice per time; points, mean; bars, SEM.

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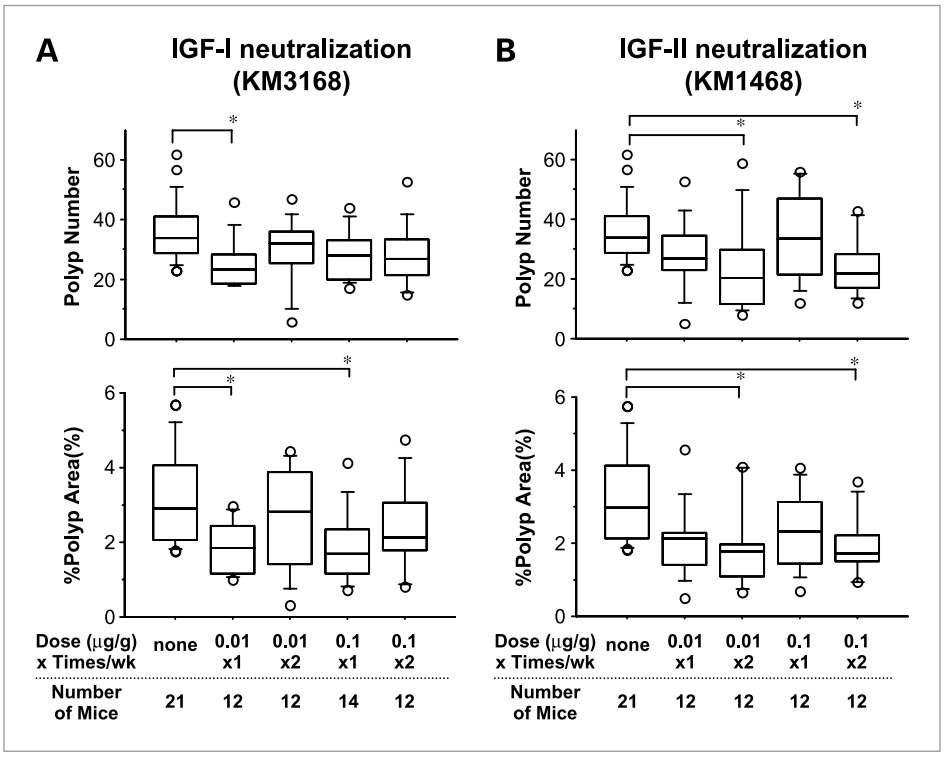

Figure 2. Effects of KM3168 or KM1468 on polyp formation in *Apc*^{1309} mice (experiment 1). Therapeutic effects of IGF-I–neutralizing antibody, KM3168 (A), and anti–IGF-II antibody, KM1468 (B), for 4 wk from 6 to 10 wk of age in *Apc*^{1309} mice (experiment 1). The box plot displays the polyp number (top) and the %polyp area (bottom) for each treatment group. The 10th, 25th, 50th (median), 75th, and 90th percentiles are shown; ○, values above the 90th and below the 10th percentiles. The polyp number and the %polyp area were significantly lower in the group given KM3168 (0.01 μg/g weight weekly) and in the group given KM1468 (0.01 μg/g weight twice weekly) compared with the control group (*, $P < 0.05$). Detailed results are given in Supplementary Table S1.

Detection of IGF Source Using Quantitative Real-time Reverse Transcription-PCR

Tissue samples from the liver, polyps of the small intestine, and adjacent normal small intestine in *Apc*$^{Min/+}$ mice (at 6, 10, and 14 wk of age, $n = 3$ at each age) and the liver and small intestine in C57BL/6J mice (at 6, 10, and 14 wk of age, $n = 3$ at each age) were rapidly deep frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from these tissues using TRIzol (Invitrogen). For the reverse transcription reaction, 1 μg of total RNA was reverse transcribed using the SuperScript II First Strand Synthesis kit (Invitrogen). The cDNA was used for amplification with FastStart Universal SYBR Green Master (Roche). Reactions were carried out in triplicate in a LightCycler 480 instrument (Roche). The expression levels of the gene were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. The primers and annealing temperatures were as follows: Igf1, TCG TCT TCA CAC CTC TTC TAC and CTT TTG TAG GCT TCA GTG GG, 60°C; Igf2, GTG GCA TCG TGG AAG AGT G and ACG GGG TAT CTG GGG AAG T, 60°C; and glyceraldehyde-3-phosphate dehydrogenase, TGT CCG TCG TGG ATC TGA C and CCT GCT TCA CCA CCT TCT TG, 60°C.

Immunostaining of IGF Signal Transduction after IGF Neutralization

The small intestine was fixed in 7% formalin, were paraffin embedded, and then cut on a microtome in 4-μm sections. Sections were deparaffinized in xylene, rehydrated in an ethanol series to PBS, and then heated in a microwave for 15 min in 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 30 min. Sections were incubated at 4°C with primary antibodies overnight. The antibodies (dilutions) applied were as follows: anti-mouse Tyr^{1161} phospho-IGF-IR antibody (1:100; Applied Biological Materials), anti-mouse IGF-IR antibody (1:50; Applied Biological Materials), anti-mouse Ser^{473} phospho-Akt antibody (1:50; Cell Signaling), anti-mouse pan-Akt antibody (1:50; Cell Signaling), anti-mouse cleaved caspase-3 antibody (1:100; Cell Signaling), and anti-Ki-67 antibody (1:50; DAKO). The sections were incubated with rabbit EnVision peroxidase-labeled polymer antibody (DAKO). Staining was completed by incubation with 3,3′-diaminobenzidine chromogen. The sections were then counterstained with hematoxylin, dehydrated, and mounted. Negative controls included incubation with PBS without the primary antibody. The proliferation index and apoptosis index were defined as the percentage of Ki-67–positive and cleaved caspase-3–positive cells, respectively, per 1,000 tumor cells (at least) in the polyp in each sample.

Statistical Analysis

Because the polyp number and %polyp area exhibited asymmetrical distribution, nonparametric tests were used. The data for polyp formation are expressed as median ± SEM, and the significance of differences between the control and the treatment groups for each variable were determined using the Mann-Whitney *U*

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test. The analyses were done using a Windows-based personal computer with the StatView package (Abacus Concepts). Significance was established at $P < 0.05$.

Results

Characterization of KM3168

Proliferation Inhibition Assay. KM3168 dose dependently inhibited the growth of HT29 cells stimulated by human and mouse IGF-I but did not inhibit the growth stimulated by human or mouse IGF-II and insulin (Fig. 1A). These results indicated that KM3168 neutralized human and mouse IGF-I but did not neutralize human and mouse IGF-II or insulin.

IGF-IR Phosphorylation Inhibition Assay. KM3168 dose dependently inhibited human and mouse IGF-I–stimulated IGF-IR phosphorylation but did not inhibit human or mouse IGF-II–stimulated or insulin-stimulated IGF-IR phosphorylation (Fig. 1B). The calculated ND_{50} of KM3168 for human and mouse IGF-I was 0.11 and 0.45 μg/mL, respectively (Fig. 1C). Based on the results of the *in vitro* assay, KM3168 and KM1468 were used as the mouse IGF-I– and IGF-II–neutralizing antibodies, respectively, for the *in vivo* studies.

Pharmacokinetics of KM3168 Antibody in Mice. The time to reach the maximum plasma concentration (T_{max}) of KM3168 and its maximum plasma level (C_{max}) were 22 hours and 90 μg/mL, respectively (Fig. 1D). After reaching the C_{max}, the plasma concentration of KM3168 decreased gradually and the half-life ($T_{1/2}$) was 168 hours. As reported previously, T_{max}, C_{max}, and $T_{1/2}$ of KM1468 were 20 to 40 hours, 50 to 55 μg/mL, and 85 hours, respectively (23).

Effects of KM3168 or KM1468 on Polyp Formation in Apc^{1309} Mice (Experiment 1)

The treatment with KM3168 at a dose of 0.01 μg/g weekly significantly reduced the total polyp number and the %polyp area of the small intestine compared with the respective control values. The total polyp numbers were 24 ± 2.4 in treated mice and 34 ± 2.3 in controls ($P = 0.003$), and the respective %polyp areas were 1.8% ± 0.2% and 2.9% ± 0.3% ($P = 0.004$; Fig. 2A). Treatment with KM1468 at the dose of 0.01 μg/g twice weekly

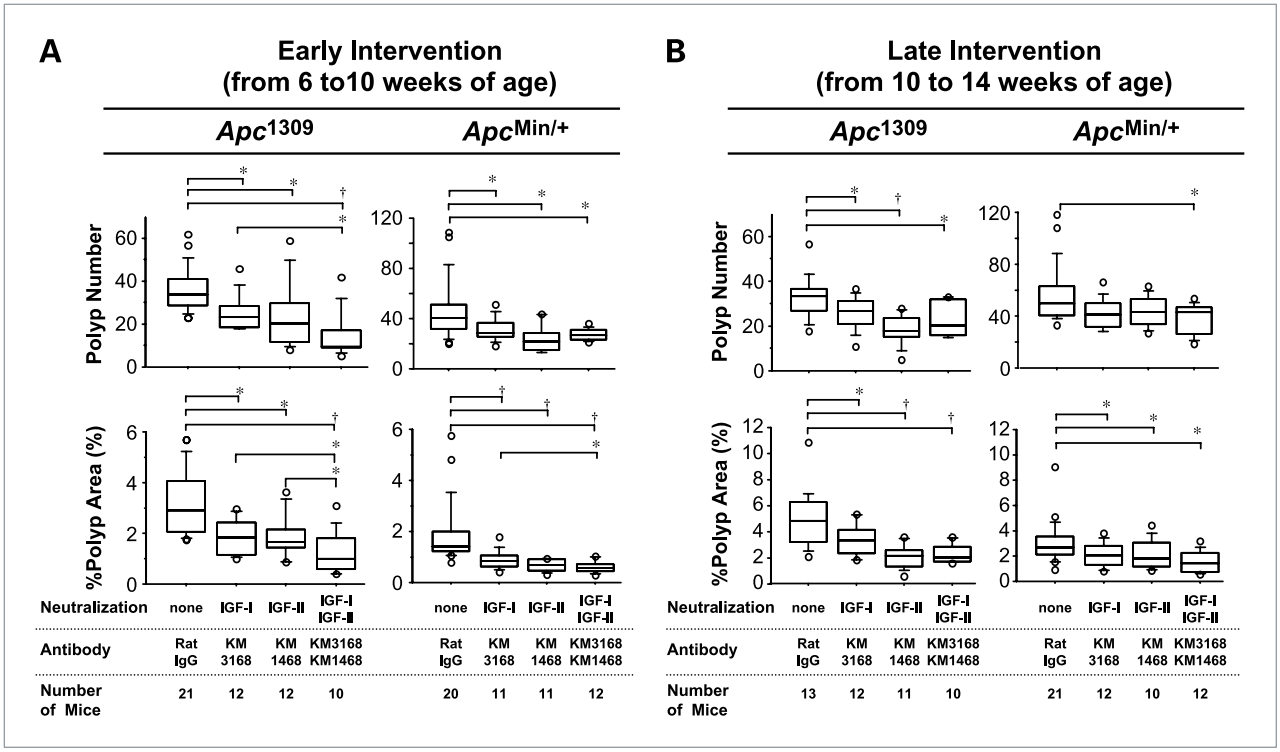

Figure 3. Effects of combined treatment with KM3168 and KM1468 on polyp formation in Apc^{1309} mice and $Apc^{Min/+}$ mice (experiment 2). The additive effects of the combined treatment of KM3168 and K1468 in Apc^{1309} mice and $Apc^{Min/+}$ mice in early and late intervention at each optimal dose were based on the results of experiment 1. The box plot displays the polyp number (top) and %polyp area (bottom) in each treatment group. The 10th, 25th, 50th (median), 75th, and 90th percentiles are shown; ○, values above the 90th and below the 10th percentiles. A, in the early intervention (from 6–10 wk of age) in Apc^{1309} mice, the total polyp number and the %polyp area were decreased more by the combined treatment with KM3168 and KM1468 than with the treatment with KM3168 alone. In $Apc^{Min/+}$ mice, the %polyp area was decreased more by the combined treatment than by the treatment with KM3168 alone. B, in the late intervention (from 10–14 wk of age), the total polyp number and the %polyp area were significantly lower in the combined treatment group than in the control group in Apc^{1309} mice and $Apc^{Min/+}$ mice (*, $P < 0.05$; †, $P < 0.001$). Detailed results are given in Supplementary Table S2.

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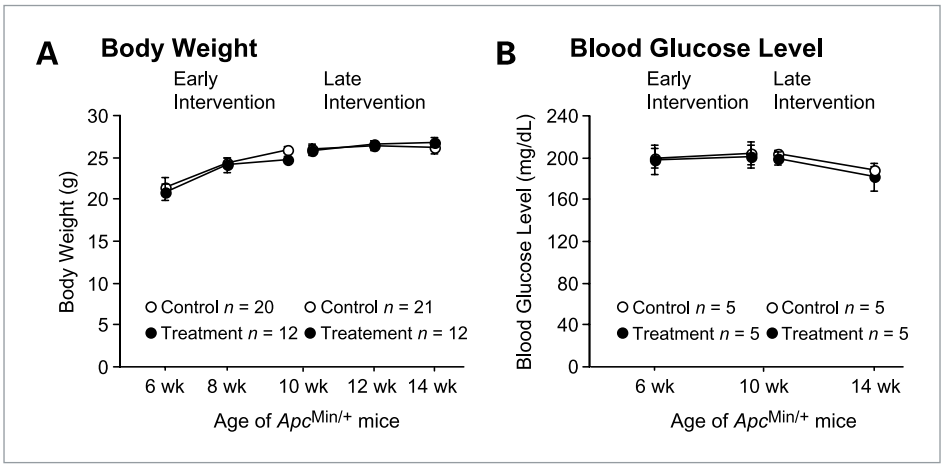

Figure 4. Effects of combined treatment with KM3168 and KM1468 on body weight and blood glucose level in $Apc^{Min/+}$ mice (experiment 2). The change in body weight (A) and blood glucose level (B) did not differ between the control group (○) and combined treatment group (●). Points, mean; bars, SEM.

significantly reduced the total polyp number and the % polyp area of the small intestine compared with the respective control values. The total polyp numbers were 21 ± 4.5 in treated mice and 34 ± 2.3 in controls ($P = 0.007$), and the respective %polyp areas were 1.7% ± 0.3% and 2.9% ± 0.3% ($P = 0.001$; Fig. 2B). Detailed results are given in Supplementary Table S1. Body weight and blood glucose level did not differ between groups in experiment 1 (data not shown).

Additive Effects of Combined Treatment with KM3168 and KM1468 on Polyp Formation in Apc^{1309} Mice and $Apc^{Min/+}$ Mice (Experiment 2)

In the early intervention study in which the antibodies were administered in Apc^{1309} mice from 6 to 10 weeks of age, the combined treatment with KM3168 and KM1468 reduced the total polyp number (10 ± 3.4 versus 24 ± 2.4, $P = 0.006$) and the %polyp area (1.0% ± 0.2% versus 1.8% ± 0.2%, $P = 0.04$) more than the treatment with KM3168 alone (Fig. 3A, left column). To confirm these results, the same study was done using $Apc^{Min/+}$ mice. The combined treatment reduced the %polyp area more than the treatment with KM3168 alone (0.6% ± 0.1% versus 0.9% ± 0.1%, $P = 0.03$; Fig. 3A, right bottom column). To assess whether IGF neutralization could reduce the number of already formed polyps, we also treated older Apc^{1309} mice and $Apc^{Min/+}$ mice from 10 to 14 weeks of age (late intervention) with the same dose as in the early intervention. In Apc^{1309} mice, the combined treatment significantly reduced the total polyp number (21 ± 2.4 versus 34 ± 2.6, $P = 0.008$) and the %polyp area (2.0% ± 0.2% versus 4.8% ± 0.6%, $P = 0.0008$) compared with the nontreated group (Fig. 3B, left column). Similarly, in $Apc^{Min/+}$ mice, only the combined treatment significantly reduced both the total polyp number (43 ± 3.5 versus 48 ± 4.6, $P = 0.002$) and the %polyp area (1.4% ± 0.3% versus 2.7% ± 0.4%, $P = 0.001$; Fig. 3B, right column). In contrast, treatment with either KM3168 or KM1468 alone significantly reduced %polyp area in Apc^{1309} and $Apc^{Min/+}$ mice but had no effect on polyp number in $Apc^{Min/+}$ mice. Detailed results are given in Supplementary Table S2. Body weight and blood glucose level did not differ between groups during experiment 2 (Fig. 4).

Source of IGFs in $Apc^{Min/+}$ Mouse

In $Apc^{Min/+}$ mice and wild-type littermate mice, the high expression levels of IGF-I mRNA in the liver were maintained throughout the experiments. IGF-I mRNA was not expressed in the intestinal polyps or in the normal small intestine (Fig. 5A). In contrast, IGF-II mRNA was highly expressed in the intestinal polyps and the normal small intestine, but scarcely in the liver (Fig. 5B). The protein expression of IGF-II was confirmed using immunostaining with anti-mouse IGF-II antibody (R&D Systems) in the polyps and the adjacent normal small intestine of $Apc^{Min/+}$ mice at 10 weeks of age (Fig. 5C).

Effects of IGF Neutralization on the Phosphorylation of IGF Signal Transduction

The combined treatment with KM3168 and KM1468 markedly decreased the phosphorylation of IGF-IR and its downstream molecule, Akt, compared with the control group, although the quantity of IGF-IR and Akt did not change (Fig. 6A and B). The treatment group had many cleaved caspase-3–positive cells in the intestinal polyps, but the control group did not (Fig. 6C). In contrast, immunoreactivity for Ki-67 did not differ significantly between the two groups (Fig. 6D). Consistent with the microscopic findings, the apoptotic index was significantly elevated in the treatment group relative to that of the control group ($P < 0.0001$; Fig. 6E), whereas there was no difference in the proliferation index between the two groups.

Discussion

Recent studies have shown the involvement of several growth factors such as epidermal growth factor and vascular endothelial growth factor in early intestinal

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tumorigenesis (30–34); however, the role of IGFs has not yet been elucidated. Ours is the study to use IGF-neutralizing antibodies to show the involvement of IGFs in intestinal polyp formation. In the first step of our study, we developed a specific antibody to both human and mouse IGF-I. The antibody KM3168 inhibited both the growth of HT29 cells and the phosphorylation of IGF-IR induced by human or mouse IGF-I but not by human or mouse IGF-II or insulin, showing the high specificity for IGF-I of the antibody. Using this antibody, KM3168, and a specific antibody for IGF-II, KM1468 (23), we showed here that blocking either IGF-I or IGF-II had a significant inhibitory effect on polyp formation in two lineages of $Apc^{+/-}$ mice. We also found that the concomitant administration of KM3168 and K1468 reduced polyp formation to a significantly greater extent than did KM3168 alone. These data suggest that IGF-I and IGF-II work together to stimulate polyp formation.

The bioavailability of IGFs is inhibited by their high-affinity binding to IGFBPs in the circulation. IGFBP proteases present in various tissues enhance the bioavailability of IGF by cleaving IGFBP bound to IGFs, which increases the free/bioactive IGF concentration within the tissue microenvironment. Matrisian et al. (35) showed that intestinal polyp formation in $Apc^{Min/+}$ mice is prevented by the absence of matrix metalloproteinase-7 (MMP-7) and by the administration of the MMP inhibitor, batimastat (36). We showed previously that MMP-7 acts as a pan-IGFBP protease (37). Because MMP-7 acts as a tissue protease, these data suggest that MMP-7 accelerates polyp formation by increasing the concentration of free/bioactive IGFs through its IGFBP protease activity in the tissue microenvironment. In this regard, we emphasize that both KM3168 and K1468 block only free/bioactive IGFs but do not affect the IGF/IGFBP complex.[4] Taken together, these data suggest the importance of free/bioactive IGFs in the tumor microenvironment.

An interesting finding in our study is that IGF-I is produced mainly in the liver, whereas IGF-II is produced in the polyp and the adjacent normal intestine. These results suggest that IGF-I acts on the tumor through an endocrine manner and that IGF-II acts mainly in an autocrine or paracrine manner in the vicinity of the tumors. These data are consistent with previous reports showing that liver-specific IGF-I deficiency reduces tumor progression in an orthotopic xenograft model (10) and that the soluble form of IGF-IIR produced constitutively in the intestine reduces intestinal polyp formation in $Apc^{Min/+}$ mice (18).

The IGF signaling pathway has received recent attention as a therapeutic target for antitumor treatment. In particular, IGF-IR is the most promising target, and many IGF-IR–targeting agents have been developed (19–21). Some of these agents are in ongoing clinical trials, and a few positive data have been obtained (38, 39). However, an IGF-IR–targeting strategy may have several disadvantages. First, because IGF-IR is distributed ubiquitously throughout the body, IGF-IR regulates multiple cellular functions, and IGF-IR blockade is expected to cause unexpected adverse events. In this regard, we note that our

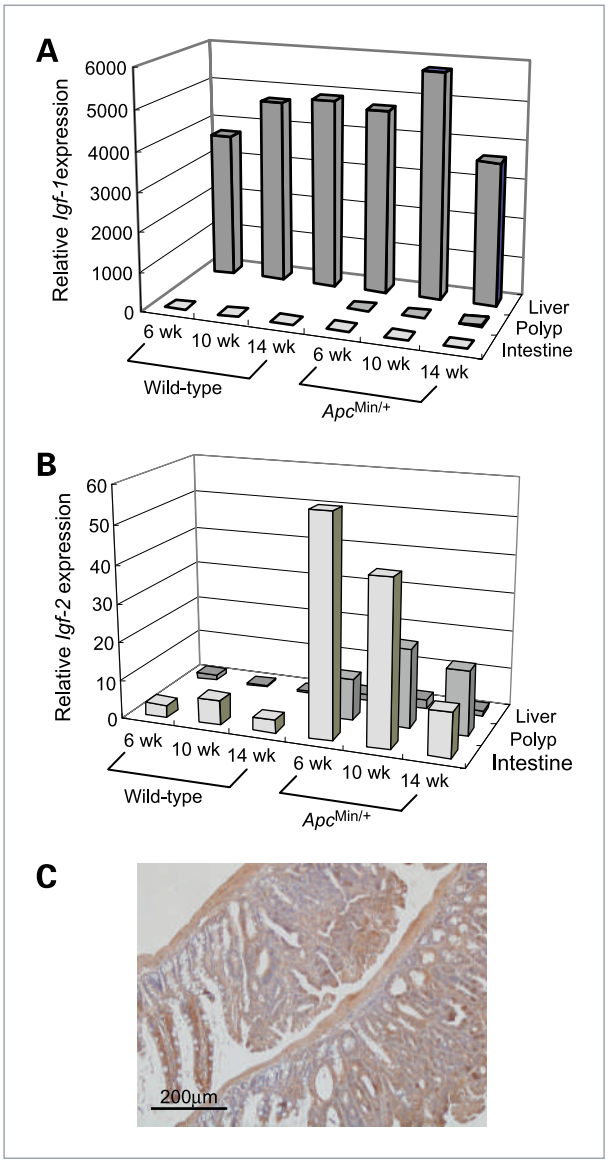

Figure 5. The source of IGFs in the $Apc^{Min/+}$ mouse. To clarify the source of IGFs in the tissues in $Apc^{Min/+}$ mice, the expression levels of IGF-I and IGF-II mRNA were examined using real-time reverse transcription-PCR. A, the high expression level of IGF-I in the liver and no expression in the small intestine or polyps suggest an endocrine action. B, in contrast, the high expression of IGF-II in the small intestine and in polyps, and almost no expression in the liver, suggest a paracrine or autocrine action. Columns, mean (n = 3 per assay). Experiments were done at least in triplicate. C, the protein expression of IGF-II was confirmed in representative sections of polyps and the small intestine in $Apc^{Min/+}$ mice by immunostaining using anti–IGF-II antibody. Bars, 200 μm.

[4] Unpublished data.

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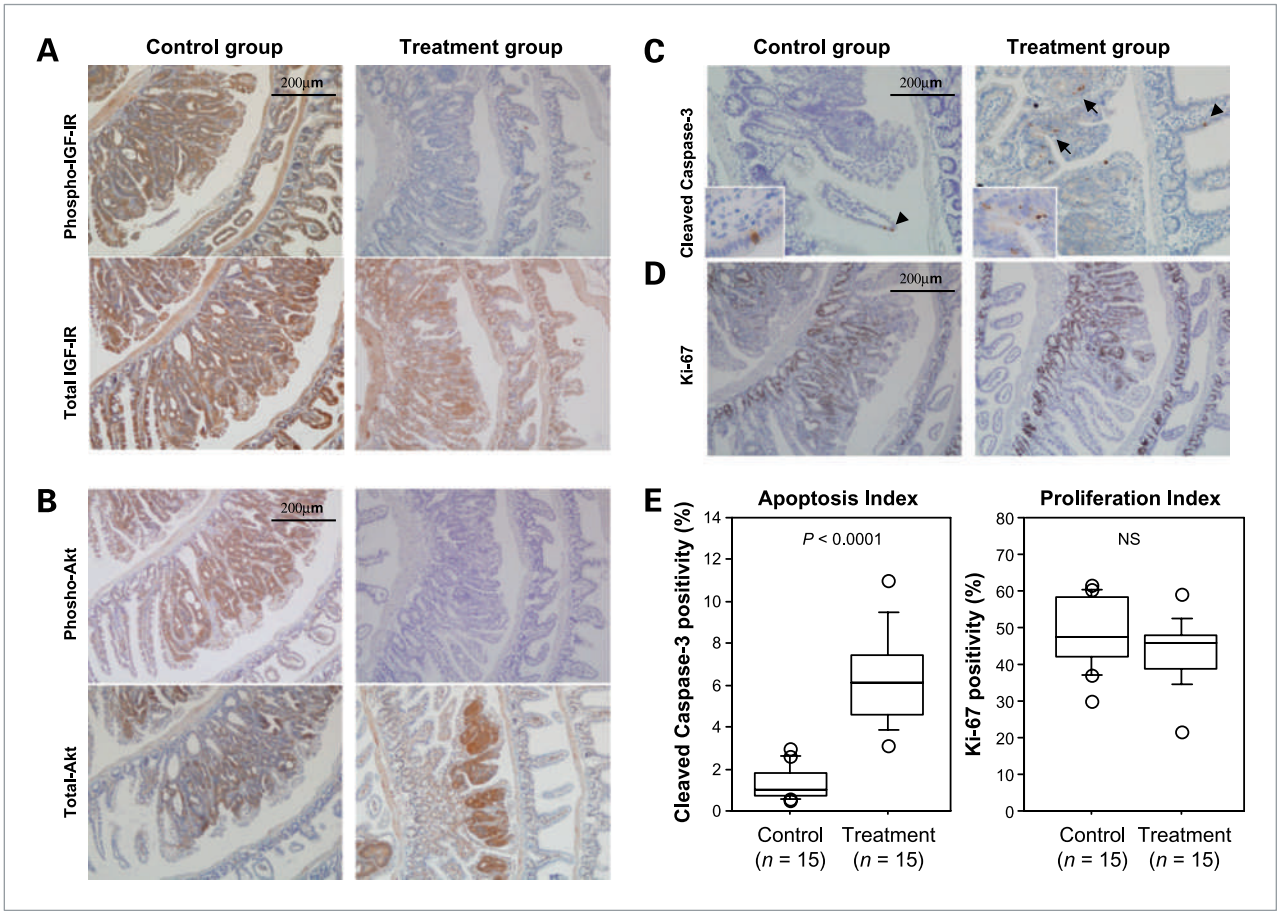

Figure 6. Effects of IGF neutralization on the phosphorylation of IGF signal transduction. Representative immunohistochemical analysis of IGF-IR signal transduction–related molecules after the combined treatment with KM3168 and KM1468 in intestinal polyps of *Apc*$^{Min/+}$ mice is shown. A and B, IGF-IR and its downstream molecule, Akt, showed lower phosphorylation levels in the treatment samples compared with the control samples. C, cleaved caspase-3–positive cells were not seen in the polyps of control samples, except for those observed in the intestinal villi, whereas positive cells were seen clearly in the polyps of the treatment samples. Insets, representative close-up pictures indicating the cleaved caspase-3–positive cells in the intestinal villi of the control samples (arrowheads) and in a polyp of the treatment samples (arrows). D, Ki-67 staining at the microscopic level did not differ between the two samples. Bars, 200 μm. E, box plot displays the apoptosis index and the proliferation index values. The 10th, 25th, 50th (median), 75th, and 90th percentiles of the variables are shown; ○, the values above the 90th and below the 10th percentiles. The apoptosis index was significantly higher in the treatment group than in the control ($P < 0.0001$, Mann-Whitney *U* test). The proliferation index did not differ between the two groups. NS, not significant.

antibodies blocked only free/bioactive IGFs and that high concentrations of free/bioactive IGFs are likely to be present predominantly in the tumor tissues because of increased IGFBP proteolysis. Thus, it is reasonable to consider that the antibodies to IGFs may have better specificity for tumors than antibodies to the IGF-IR. Second, IGF-IR has a homology with the insulin receptor; IGF-IR–blocking agents may cross-react with the insulin receptor and thus induce glucose intolerance (38–41). However, because our antibodies do not cross-react with insulin *in vitro*, they are not expected to affect the blood glucose level. In fact, we observed a slight decrease of plasma level of insulin 1 week after the treatment; however, blood glucose level remained unchanged under our experimental conditions (most therapeutic-effective dose, i.e., 0.01 μg/g of KM3168 weekly and 0.01 μg/g of KM1468 twice weekly; Supplementary Fig. S1A).

We also found no dose dependency of IGF-neutralizing antibodies in inhibiting intestinal polyp formation. More remarkably, we observed that high doses of IGF-neutralizing antibody did not have a therapeutic effect on intestinal polyp formation, although the reason for this lack of effect of high doses of IGF-I antibody on intestinal polyp formation is unclear at present. Interestingly and unexpectedly, however, we found in the preliminary experiment that administration of a high dose of IGF-I–neutralizing antibody (10 μg/g weight) increased the plasma IGF-I concentrations.[5] Thus, one may speculate that plasma IGF-I is depleted rapidly after an excess administration of IGF-I–neutralizing

[5] Unpublished data.

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antibody, which may increase the secretion of growth hormone through a feedback mechanism and eventually increase the plasma IGF-I concentration. This possible mechanism might be responsible for the failure of high-dose antibody administration in reducing polyp formation. Plasma levels of growth hormone and IGF-I slightly decreased 1 week after the treatment, whereas that of IGF-II remained unchanged under our experimental conditions (Supplementary Fig. S1B).

In this study, we showed that IGF-neutralizing antibodies mainly enhanced the induction of apoptosis *in vivo*, but this treatment did not seem to affect the proliferation of intestinal polyps as evaluated by the immunostaining method. These results are consistent with earlier reports involving IGF-IR–targeting therapy (24, 42) and suggest that IGFs act mainly as antiapoptotic factors in the intestinal tumorigenesis process. Because IGF-neutralizing antibodies alone do not have a drastic effect on intestinal tumorigenesis, however, it may be necessary to combine them with chemotherapy or radiation therapy similar to IGF-IR–targeting therapy (43, 44) or to limit the applications to highly IGF-dependent tumors (45). The combination therapy of IGF-neutralizing antibodies with an MMP inhibitor (36) or cyclooxygenase-2 inhibitor (46), both of which have inhibitory effects on intestinal polyp formation, is also attractive.

Although KM1468 does not cross-react with mouse IGF-I, it can neutralize both human IGF-I and IGF-II, and is expected to be applied in humans. In this regard, we note that the plasma IGF-I/-II ratio in humans differs significantly from that in rodents. In humans and rodents, IGF-I is expressed at relatively high levels throughout life, and its plasma concentration is similar in the two species. In contrast, IGF-II expression becomes attenuated in most tissues in rodents and persists at negligible levels in adulthood, whereas in humans, IGF-II expression remains relatively high throughout life. Therefore, the plasma concentration of IGF-II is higher in humans (700 ng/mL) than in rodents (20 ng/mL), particularly in adults. This difference will be a considerable barrier to applying the results of this mouse model directly to humans. To overcome this limitation, the IGF-neutralizing antibody treatment must be evaluated in models with larger mammals whose plasma distribution of IGF-I and IGF-II is similar to humans.

In conclusion, our present study showed that IGF-I and IGF-II play important roles in polyp formation in $Apc^{+/-}$ mice. The antibodies specific to IGF-I and IGF-II, KM3168 and KM1468, seem to be promising therapeutic tools as antitumor agents. However, further studies are required before applying these antibodies to humans because the efficacy of their actions against human tumors cannot be extrapolated directly from the data obtained in murine models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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