Blockade of Paracrine Supply of Insulin-Like Growth Factors Using Neutralizing Antibodies Suppresses the Liver Metastasis of Human Colorectal Cancers

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Abstract

Environmental stimuli, such as organ-specific growth factors, can influence the metastatic potential of a tumor. The liver is the main source of insulin-like growth factors (IGFs). The importance of IGF signal in hepatic metastasis has been clarified mainly by IGF-I receptor targeting strategies. This study aims to confirm these precedent reports by novel tool, neutralizing antibodies against IGFs and to show that IGFs are promising therapeutic targets for hepatic metastasis in vivo. Hepatic metastasis was induced by intrasplenic injection of human colorectal cancer cell line, HT29. The antitemetastatic effects of three antibodies (anti-mouse IGF-I, anti-mouse IGF-II, and anti-human/mouse IGF-II designated KM1468) were tested singly or in combination in the early phase of metastasis. The dose escalation effect of KM1468 and its survival benefit were examined in the early and late phases of metastasis. The mechanism of IGF neutralization was investigated with immunohistochemistry. Dual neutralization of paracrine IGF-I and IGF-II showed modest additive antitemetastatic effects than single neutralization of IGF-I or IGF-II. In any phase of metastasis, neutralization led to significant tumor growth inhibition and longer survival. Dose escalation of KM1468 influenced survival only in the late phase of metastasis. Apoptosis increased significantly in the antibody-treated group compared with the control group (P = 0.0025). In conclusion, IGFs are promising therapeutic targets for hepatic metastases of colorectal cancers. However, the IGF dependency is probably variable in the metastatic process.

Almost one-third of patients dying from colorectal cancer have metastatic tumors only in the liver. The control of hepatic metastasis leads directly to an improvement in the overall cure rate for colorectal cancer (1). However, our lack of understanding of the molecular mechanisms mediating the process of hepatic metastasis means that few theoretical treatments for this syndrome have yet been developed.

The insulin-like growth factors (IGFs) have been widely investigated for a possible role in promoting the oncogenic transformation, growth, and survival of cancer cells (2–6). They are expressed ubiquitously and act as endocrine, paracrine, and autocrine growth factors. In most tissues, they are synthesized together with six molecular species of specific binding proteins (IGFBP-1 to IGFBP-6), which modulate IGF action in the cell environment, generally by inhibiting it. Limited proteolysis of IGFBPs is an essential mechanism in the regulation of IGF bioavailability both in the bloodstream and at the cellular level (7, 8). The liver is the main source of IGFBPs, and there are reports demonstrating the importance of IGF signals in hepatic metastasis formation (9–13). We speculated in a previous report that free/bioactive IGFs generated from IGFBP-3 proteolysis by matrix metalloproteinase-7 play a crucial role in hepatic metastasis in human colorectal cancers (14).

Recently, this IGF system has been recognized as a potent therapeutic target for several cancers (15–17). IGF-I receptor (IGF-IR), in particular, is the most attractive target and many IGF-IR targeting strategies have been developed in preclinical settings. These include inhibitory antibodies (18–20), a soluble form of the dominant-negative receptor (21–24), and small molecular compounds that act as tyrosine kinase inhibitors (25–28). On the other hand, little has been reported to date on IGF-ligand targeting strategies. In this study, we clarified the participation of the IGF paracrine signal (both IGF-I and IGF-II) in the hepatic metastasis of colorectal cancers using anti-host/ mouse IGF-neutralizing antibodies in vivo. This is the first report showing the possible use of a novel growth factor-targeting strategy for hepatic metastases of colorectal cancers and shows the importance of understanding the host-tumor interaction in the local tumor area.
Materials and Methods

**Proteins and reagents.** The commercially available antibodies used in this study were goat polyclonal antibody against mouse IGF-I and mouse IGF-II (R&D Systems, Inc., Minneapolis, MN), rabbit polyclonal antibody against human carcioembryonic antigen (CEA; DAKO, Glostrup, Denmark), mouse monoclonal antibody against Ki-67 (DAKO), rabbit polyclonal antibody against cleaved caspase-3 (Cell Signaling Technology, Beverly, MA), and rabbit monoclonal antibody against factor VIII antigen (Zymed Laboratories, Inc., San Francisco, CA). Normal goat IgG was purchased from R&D Systems. KM1468 (rat IgG2b with Fc fragment) was raised by immunizing a rat with purified full-length human IGF-I protein. This monoclonal antibody was selected for its ability to neutralize the biological activity of human IGF-I, human IGF-II, and mouse IGF-II. It does not neutralize the biological activity of mouse IGF-I. This antibody shows no cross-reactivity with insulin on direct ELISA. KM1762 (rat IgG2a with Fc fragment) was raised by immunizing a rat with purified full-length avermectin protein as a control for IGF. Detailed characterization of KM1468 has been published elsewhere (29).

**Animals.** Male severe combined immunodeficient mice, 6 weeks old, were purchased from CLEA Japan, Inc. (Tokyo, Japan) and maintained at the National Cancer Center Research Institute East (Chiba, Japan) under specific pathogen-free, temperature-controlled air conditions throughout this study according to institutional guidelines. Cages, bedding, and drinking water were autoclaved. Food was sterilized by irradiation. The mice used in all experiments were 8 to 12 weeks old, with 20 to 25 g body weight.

**Cell lines and cell culture.** HT29 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum and grown to 80% confluence. The cells were washed with PBS, trypsinized (0.05% trypsin-0.02% EDTA), and harvested from the dishes by pipetting. After centrifugation at 400 x g for 1 minute at 4°C, pellet was resuspended in PBS and the cell concentration was adjusted to 1 x 10^7 cells/mL. Only single cell suspensions with a viability of >90% (determined by trypsin blue exclusion) were used for injection to produce hepatic metastasis.

**Induction of hepatic metastasis.** This procedure was done as described previously (30). Each mouse was anesthetized by ether inhalation and its abdomen was sterilized with alcohol swabs. The spleen was carefully exteriorized by a midline incision and tumor cell suspension (2 x 10^6 cells, 200 µL) was injected subserosa with a 27 gauge needle. After allowing 3 minutes for the tumor cells to enter the portal circulation, a splenectomy was done; bleeding was stopped with a heat probe. Following the splenectomy, the remaining fascia was replaced in the abdominal cavity and the abdominal muscles and skin were sutured separately with 7/0 and 5/0 nylon (Bear Medic Corp., Tokyo, Japan), respectively.

**Measurement of tumor burden.** We analyzed the tumor burden in the liver by histomorphometrically examining sections stained for CEA. The livers were removed and divided into four parts according to anatomic segment (i.e., into the left, middle, right, and posterior lobes). All samples were fixed in 30% buffered formalin overnight and embedded in paraffin. Paraffin sections (5 µm) of the maximal cut surface in each block were cut. Immunohistochemical staining was done with DAKO EnVision Plus System HRP according to the established protocol. Anti-CEA antibody was used at a dilution of 1:300 and no antigen retrieval was required. Tumor burden was defined as the total CEA-positive area in all four lobes (in mm^2) calculated with the KS300 computed image analysis system version 3.00 (Carl Zeiss, Oberkochen, Germany).

**Carcinoembryonic antigen measurement in mouse sera.** Blood samples were taken from retro-orbital venous plexus of mice 2 and 4 weeks after intrasplenic injection of tumor cells. The samples were centrifuged at 1,000 x g for 5 minutes and human CEA was measured in serum samples with a chemiluminescence immunoassay (31) using the automated Architect i2000 system (Abbott Laboratories, Abbott Park, IL).

**Experimental design for anti–insulin-like growth factor antibody treatment in vivo.** KM1468, KM1762, and goat IgG solutions were prepared with sterile PBS and stored at −80°C until use. These substances were given intraperitoneally (i.p.) into severe combined immunodeficient mice according to the following two experimental designs.

**Experiment 1:** Estimation of the effect and adverse event of anti-IGF antibody treatment. This study was designed to show the response to anti-IGF antibody treatment and to clarify which ligand, IGF-I or IGF-II, is more critical for hepatic metastasis. Each antibody (0.1 µg/g body weight) was given i.p. into male severe combined immunodeficient mice at weekly intervals starting on the day of the intrasplenic injection of cancer cells. This experiment represents the prevention of metastasis or the treatment of micrometastasis. To estimate the major systemic adverse event of anti-IGF treatment, body weight (weekly) and blood glucose level (biweekly) were monitored. All mice were sacrificed at 4 weeks after intrasplenic injection of cancer cells. Table 1 explains the protocols of experiment 1.

![Table 1. Protocols of experiment 1](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Antibodies</th>
<th>Dose (µg/g)</th>
<th>Neutralization</th>
<th>Treatment schedule</th>
</tr>
</thead>
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<tr>
<td>A</td>
<td>11</td>
<td>Goat IgG</td>
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<td>none</td>
<td>Ab i.p. 0 1W 2W (CEA/BG) 3W 4W (CEA/BG) Sacrifice</td>
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<tr>
<td>B</td>
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<td>mIGF-I</td>
<td>Cancer Cells</td>
</tr>
<tr>
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<td>0.1</td>
<td>mIGF-II</td>
<td></td>
</tr>
<tr>
<td>D</td>
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</table>

Ab abbreviations: Ab, Antibody; i.p., intraperitoneal administration; BG, Blood Glucose.

* mIGF-I, mouse IGF-I.

1 mIGF-II, mouse IGF-II.

hIGF-II, human IGF-II.
Experiment 2: Modification of the treatment regimen and estimation of the survival benefit of anti-IGF antibody treatment. This study was designed to show the effects of specific doses of KM1468 and different starting points of the treatment. The late-starting experiment (groups D and E) represents treatment of established metastatic tumors. The survival benefit of each therapeutic regimen was evaluated in HT29 cells after 6-week follow-up periods, respectively. Reproducible results that almost all mice died of tumor progression within 6 weeks were obtained; therefore, these experiments were terminated at this point. Table 2 explains the protocols of experiment 2. A dose escalation analysis of anti-mouse IGF-I antibody cannot be done for economical reasons.

Table 2. Protocols of experiment 2

<table>
<thead>
<tr>
<th>Group</th>
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<th>Antibodies</th>
<th>Dose (µg/g)</th>
<th>Treatment schedule</th>
<th>Abbr. of Regimen</th>
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<td></td>
<td>control</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KM1762</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>12</td>
<td>Anti mIGF-I</td>
<td>0.1</td>
<td>Early/Low (= Experiment 1)</td>
<td>KM1468 0.1</td>
</tr>
<tr>
<td>C</td>
<td>12</td>
<td>Anti mIGF-I</td>
<td>0.1</td>
<td>Early/High</td>
<td>KM1468 1.0</td>
</tr>
<tr>
<td>D</td>
<td>12</td>
<td>Anti mIGF-I</td>
<td>0.1</td>
<td>Late/Low</td>
<td>KM1468 0.1</td>
</tr>
<tr>
<td>E</td>
<td>14</td>
<td>Anti mIGF-I</td>
<td>0.1</td>
<td>Late/High</td>
<td>KM1468 1.0</td>
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</table>

Abbreviations: Ab, Antibody; i.p., intraperitoneal administration; BG, blood glucose.
*mIGF-I, mouse IGF-I.

Fig. 1. Therapeutic effects of anti-IGF antibodies on the early treatment model (experiment 1). A box plot displays the tumor burden (mm²) of each treatment group. The 10th, 25th, 50th (median), 75th, and 90th percentiles of the variables are shown, and values above the 90th and below the 10th percentiles are plotted as open circles. The tumor burden was significantly decreased in groups B, D, and E compared with that of group A (P = 0.035, 0.014, and 0.020, respectively, Mann-Whitney U test).

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mouse</th>
<th>Neutralization</th>
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<td>239</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>mlIGF-I</td>
<td>142</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td>mlIGF-II</td>
<td>140</td>
</tr>
<tr>
<td>D</td>
<td>11</td>
<td>mlIGF-I</td>
<td>88</td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>mlIGF-II</td>
<td>111</td>
</tr>
</tbody>
</table>
Fig. 2. Macroscopic appearances and CEA staining of liver metastasis after anti-IGF antibody treatment in experiment 1. Metastatic tumor cells were identified as whitish and patchy areas, especially at the margin of the liver. Macroscopic findings showed that dual neutralization of IGF-I and IGF-II (groups D and E) had more potent antimetastatic effects than did the single neutralization of IGF-I (group B) or IGF-II (group C). Objective evaluation of tumor burden was done based on the CEA staining area as described in Materials and Methods. Group A, control antibody; group B, anti-mouse IGF-I antibody alone; group C, anti-mouse IGF-II antibody alone; group D, anti-mouse IGF-I antibody + anti-mouse IGF-II antibody; group E, anti-mouse IGF-I antibody + KMI468. Detailed treatment schedules are shown in Table 1. *Lt, left; *Mid, middle; *Rt, right; *Post, posterior.

Fig. 3. Effect on body weight (A) and blood glucose level (B) of anti-IGF antibody treatment. No significant differences in the change of body weight and blood glucose level were found between the control group (group A of experiment 1, n = 11, open circle) and the anti-IGF antibody-treated group (group E of experiment 1, n = 11, closed circle). Points, mean; bars, SE.
Immunohistochemistry of tumor sections. Proliferating cells were detected by Ki-67 staining, apoptosis was identified by cleaved caspase-3-positive cells, and angiogenesis was defined by microvessel density (MVD) based on factor VIII staining. Immunohistochemical staining was done as described above. Anti-Ki-67 and anti-cleaved caspase-3 antibodies were used at dilutions of 1:50 and 1:200, respectively. Microwaving (95°C, 20 minutes) in citrate buffer (pH 6.0) was required for antigen retrieval. The proliferation index (PI) and apoptosis index (AI) 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 most highly stained area in each sample. The necrotic area was not included in the evaluation of apoptosis or angiogenesis. Anti-factor VIII antibody is ready to use and requires no dilution; trypsin digestion (37°C, 30 minutes) was required for antigen retrieval. Factor VIII–stained sections were scanned at low magnification (×40) to determine areas with the highest numbers of microvessels (hotspots). Microvessels were counted at a magnification of ×200 in three hotspots on each section and MVD was calculated as the average of the three measurements. All immunohistochemical studies included a negative control in which the primary antibody was omitted.

Statistical analysis. Because the tumor area, serum CEA levels, PI, AI, and MVD values exhibited asymmetrical distributions, nonparametric tests were used. The statistical significance of differences in each variable between the control and the treatment groups was determined with the Mann-Whitney U test. The relationship between tumor area and serum CEA levels was assessed by linear regression analysis. Survival curves were graphed using calculations based on the Kaplan-Meier method. Survival differences between the two groups were assessed by the log-rank test. Statistical calculations were done on a Macintosh personal computer with the StatView package (Abacus Concepts, Berkeley, CA). Statistical significance was established at P < 0.05.

Results

Effect of anti-insulin-like growth factor antibodies on the early phase of metastasis (experiment 1). The results of experiment 1 are shown in Fig. 1. Neutralization of IGF-I significantly decreased the tumor burden (group A versus group B; \( P = 0.035 \)). Neutralization of IGF-II was marginally effective in inhibiting the development of hepatic metastasis (group A versus group C; \( P = 0.053 \)). Not significantly different, however, neutralization of both IGF-I and IGF-II in combination shows the modest additive effect compared with single neutralization of IGF-I or IGF-II (group B versus group D; \( P = 0.36 \); group C versus group D; \( P = 0.21 \)). The antimetastatic effect of KM1468 was equivalent to that of commercially available antibody (group D versus group E; \( P = 0.58 \)). The macroscopic appearances and CEA staining of representative mouse from each group are shown in Fig. 2.

Estimation of adverse event in anti-insulin-like growth factor antibody-treated mice (experiment 1). Body weight (weekly; Fig. 3A) and blood glucose level (biweekly; Fig. 3B) were

![Graph showing correlation between tumor burden (mm²) and serum CEA levels (ng/ml).](https://www.aacrjournals.org/files/10.1158/1078-0432.CCR-04-1149-Fig-4.jpg)

**Fig. 4.** Correlation between tumor burden (mm²) and serum CEA levels (ng/ml). Serum CEA levels correlate significantly with tumor burden (\( R^2 = 0.832, P < 0.0001 \), linear regression analysis), indicating that serum CEA is a useful tumor marker in this animal model.

![Box plot showing therapeutic effects of anti-IGF antibodies according to treatment regimen (experiment 2).](https://www.aacrjournals.org/files/10.1158/1078-0432.CCR-04-1149-Fig-5.jpg)

**Fig. 5.** Therapeutic effects of anti-IGF antibodies according to treatment regimen (experiment 2). A box plot displays the serum CEA levels (ng/ml) at 4 weeks after intrasplenic tumor injection. The 10th, 25th, 50th (median), 75th, and 90th percentiles of the variables are shown, and the values above the 90th and below the 10th percentiles are plotted as open circles. The serum CEA levels were significantly lower in groups B, D, and E than in group A (\( P = 0.012, 0.046, \) and 0.018, respectively, Mann-Whitney U test).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Early/Low</th>
<th>Early/High</th>
<th>Late/Low</th>
<th>Late/High</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>16</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>B</td>
<td>369</td>
<td>144</td>
<td>313</td>
<td>247</td>
<td>172</td>
</tr>
</tbody>
</table>

CEA (median: ng/ml)
analyzed in group A (control group, \(n = 11\)) and group E (anti-mouse IGF-I antibody + KM1468 treatment group, \(n = 11\)) of experiment 1. No differences in body weight and blood glucose level were noted between the two groups during 4 weeks of treatment. It is not known exactly why hypoglycemia was associated with disease progression (Fig. 3B).

**Correlation between tumor burden and serum carcinoembryonic antigen levels.** A good correlation \((R^2 = 0.832; P < 0.0001)\) was observed between tumor burden and serum CEA level (Fig. 4). Based on this result, serum CEA levels were substituted for tumor area in assessing the objective response to therapeutic interventions in experiment 2.

**Modification of treatment regimen (experiment 2).** Figure 5 shows the difference in therapeutic effect according to treatment regimen. Group B in experiment 2 confirmed the reproducibility of experiment 1. Neutralization of IGF had both a preventive effect on metastasis (group A versus group B; \(P = 0.012\)) and a growth inhibitory effect on the established metastatic tumor (group A versus group D; \(P = 0.046\); group A versus group E; \(P = 0.018\)). However, it is unclear why this treatment was not effective in group C (group A versus group C; \(P = 0.39\)). Moreover, no dose escalation effect of KM1468 on the response was observed in either the early or the late treatment models.

**Survival benefit of anti-insulin-like growth factor antibody-treated tumor sections.** To understand the processes by which tumor growth is inhibited by anti-IGF antibodies in vivo, histologic analysis was done on tumor sections from group A (control group, \(n = 11\)) and group E (anti-mouse IGF-I antibody + KM1468 treatment group, \(n = 11\)) of experiment 1. Figure 7A shows H&E staining, Ki-67 staining, cleaved caspase-3 staining, and factor VIII staining. The PI, AI, and MVD values in these tumor sections were defined and quantified manually as described in Materials and Methods. Cleaved caspase-3 immunohistochemistry is more specific and convenient than terminal deoxynucleotidyl transferase–mediated nick end labeling analysis for detecting the apoptotic cells in tissue sections (32, 33). Numerous apoptotic bodies were apparent in H&E-stained sections from the antibody-treated group compared with those from the control group (Fig. 7A). Numbers of Ki-67-positive cancer cell nuclei and factor VIII–positive microvessels were similar in the two groups. Consistent with the microscopic findings, there was no difference in PI or MVD values between the control and the antibody-treated groups, whereas the AI was significantly elevated in the antibody-treated group relative to that of the control group \((P = 0.0025; \text{Fig. 7B})\).

**Discussion**

The complex interaction between tumor cells and the host microenvironment can significantly influence tumor progression.
A tumor cell that has implanted at a distant site to form a viable metastatic lesion must be capable of responding appropriately to environmental stimuli, such as organ-specific growth factors. Epidermal growth factor (35–37), hepatocyte-derived growth factor (38, 39), and IGFs (9, 12, 13) are growth factor ligands implicated in the growth of hepatic metastases of colorectal cancers. We have confirmed that the levels of epidermal growth factor had levels of apoptotic bodies (arrows; ×200) noticeably higher than those of the controls (H&E staining). No microscopic difference was observed in Ki-67 staining (×200) or factor VIII staining (×100) between the two groups, whereas extensive cleaved caspase-3-positive cells were apparent in the antibody-treated sections (×200). Proliferation index (PI) and microvessel density (MVD) did not differ between the two groups.

Fig. 7. Histologic analysis of metastatic liver tumor sections from the control group (group A of experiment 1, n = 11) and the anti-IGF antibody-treated group (group E of experiment 1, n = 11). Tumor samples were resected, fixed, and processed for H&E, anti-Ki-67, anti-cleaved caspase-3, or anti-factor VIII immunohistochemistry.

A, antibody-treated samples had levels of apoptotic bodies (arrows; ×200) noticeably higher than those of the controls (H&E staining). No microscopic difference was observed in Ki-67 staining (×200) or factor VIII staining (×100) between the two groups, whereas extensive cleaved caspase-3-positive cells were apparent in the antibody-treated sections (×200). B, box plot displays PI, AI, and MVD values. The 10th, 25th, 50th (median), 75th, and 90th percentiles of the variables are shown, and the values above the 90th and below the 10th percentiles are plotted as open circles. AI was significantly higher in the antibody-treated group than in the control (P = 0.0025, Mann-Whitney U test). PI and MVD did not differ between the two groups. Abs, Antibodies; NS, not significant; HPF, high-power field (×200).
Neutralization of Paracrine IGFs Suppresses the Liver Metastasis

In conclusion, paracrine IGFs present a novel molecular target for therapeutic strategies directed against hepatic metastases of colorectal cancers. Neutralization of IGF by ligand-specific antibodies is the first strategy in attacking the host-derived growth factors activated by the tumor. More attention should be focused on the host-tumor interaction in the tissue microenvironment when molecular targeting therapies are designed. Further studies are required to clarify the regulation of IGF dependency in the process of hepatic metastasis.

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References
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