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主論文(1)

Bioactive insulin-like growth factors as a possible molecular target for non-islet cell tumor hypoglycemia

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Abbreviations

NICTH; non-islet cell tumor hypoglycemia, IGF; insulin-like growth factor, IGF-1R; IGF type 1 receptor, IR; insulin receptor, IGFBP; IGF binding protein, HMW IGF-2; high molecular weight IGF-2, KIRA; the kinase receptor activation assay

Abstract

Non-islet cell tumor hypoglycemia (NICTH) is a paraneoplastic syndrome characterized by persistent, severe hypoglycemia with a wide variety of solid tumors. It is considered to cause hypoglycemia by increasing the insulin-like bioactivity of the circulating insulin-like growth factor (IGF) system, however, the precise mechanism of hypoglycemia remains unclear. In this manuscript, we report on a patient suffering from NICTH caused by a small cell carcinoma of the colon. This is the first report focusing on the role of bioactive IGFs for this pathological condition. First, we demonstrated that the IGF signal pathway has been activated in this tumor in an autocrine and/or paracrine manner using immunohistochemical analysis. Second, we confirmed that bioactive IGFs in the patient's serum were increased using a modified kinase receptor activation assay, thus bioactive IGFs (mainly IGF-2) could be considered to play a major pathogenic role in enhanced hypoglycemic insulin-like activity. Third, increased IGF bioactivity in the patient's serum was completely inhibited by an anti-IGF neutralizing antibody *in vitro*. These results suggest that neutralization of bioactive IGFs might become a novel therapeutic strategy for NICTH to relieve the hypoglycemic symptoms together with the tumor suppressive effect.

Key words

Non-islet cell tumor hypoglycemia (NICTH), Insulin-like growth factor (IGF-1), Insulin-like growth factor-2 (IGF-2), bioactive IGF, free IGF, Kinase receptor activation assay (KIRA), Anti-IGF neutralizing antibody,

Introduction

Insulin-like growth factor (IGF)-1 and IGF-2 are ligands for IGF type 1 receptor (IGF-1R), which is a cell-surface tyrosine kinase signaling receptor, and for insulin receptor (IR). The physiological activities of IGFs are modulated by six IGF binding proteins (IGFBP-1 through -6) that may inhibit the signaling pathway by capturing free IGFs, thus blocking receptor binding.[1,2] Therefore, receptors are only activated by free/bioactive IGFs released from the IGF-IGFBP complex, mainly by proteolysis of IGFBPs.[1,2] Recently, it has been recognized that these receptors are widely expressed on neoplastic tissues, and overexpression of ligands, particularly IGF-2, is common in many malignancies.[1]

Non-islet cell tumor hypoglycemia (NICTH) is a paraneoplastic syndrome characterized by persistent, severe hypoglycemia with a wide variety of tumor types that are either of mesenchymal or epithelial origin, and should be suspected in any patient with hypoglycemia without clear etiology.[3–6] It is associated with secretion of incompletely processed IGF-2 (high molecular weight (HMW) IGF-2) by the tumor into the circulation, and some reports attribute the etiology of NICTH to the increasing insulin-like activity of systemic IGF, mainly HMW IGF-2.[3–9] Thus, the mechanism of hypoglycemia has been partially elucidated by previous studies, but is still not sufficiently well understood.[3–9] Therapies that are directed at reduction of the tumor burden should theoretically improve NICTH.[3-6] However, because many NICTH causing tumors are found in the advanced stage, when surgical resection is no longer possible, we have no choice but to perform systemic chemotherapy, often with very limited success.[3–5]

Here, we report a patient with NICTH caused by an advanced small cell carcinoma of the colon, focusing on the role of bioactive IGFs for this pathological condition and the possibility of bioactive IGFs being a novel therapeutic target for NICTH.

Case Report

A 54-year-old male required emergency admission to the hospital due to a loss of consciousness. On admission, severe hypoglycemia was noted with a blood glucose level of 20 mg/dl. Computed tomography of the abdomen revealed multiple tumors in the liver (Figure 1A) and thickening of the ascending colonic wall (Figure 1B, arrow). A total colonoscopy demonstrated a large ulcerated and circumferential tumor at the ascending colon (Figure 1B, inset). Biopsy specimens from the primary and liver tumors revealed that the tumor cells were composed of highly atypical small cells with hyperchromatic nuclei and scanty cytoplasm (Figure 2A). Immunohistochemistry was performed using anti-synaptophysin antibody (DAKO, Carpinteria, CA, USA), anti-Ki67 antibody (DAKO). The tumor cells were strongly positive for synaptophysin, and the Ki-67 labeling index was over 70% (Figure 2A). Based on these findings, the colonic tumor was diagnosed as a small cell carcinoma with multiple liver metastases (Neuroendocrine cell carcinoma, small-cell type, WHO classification 2010). Moreover, immunostaining was also performed using anti-IGF-2 antibody (ab9574, Abcam, Cambridge, UK), anti-IGF-1R antibody (Ab 1161, Applied Biological Materials, Richmond, BC, Canada), and anti-phospho-specific IGF-1R antibody (Phospho-Tyr 1161, Applied Biological Materials). The tumor cells were positive for IGF-2, IGF-1R, and phosphorylated IGF-1R, suggesting that the IGF signal in this tumor was activated in an autocrine and/or paracrine manner (Figure 2B).

The laboratory data on admission are summarized in Table 1. Decreased levels of insulin, C-peptide, IGF-1, and growth hormone, together with a normal level of total IGF-2, are typical laboratory data for NICTH.[3–5] HMW IGF-2 could only be detected in the patient's serum by western blot analysis (Figure 3A). With these laboratory data, the

cause of the severe hypoglycemia was diagnosed as NICTH caused by an advanced neuroendocrine cell carcinoma of the colon.

To evaluate bioactive IGFs in the circulation, we developed the novel bioassay directed against the human phosphorylated IGF-1R using gel blot analysis as a modification of the kinase receptor activation assay (KIRA) reported by Chen *et al*.[10] Briefly, subconfluent serum-free embryonic mouse hypothalamic (N7) cells overexpressing the human IGF-1R (N7-IGF-1R cells) were stimulated by a diluted blood sample (1mg protein) in Krebs–Ringer bicarbonate buffer (protein concentration was adjusted to 1 mg/mL) and incubated for 15 min. Each sample (10 μg of cell lysate) was fractionated by 7.5% SDS-PAGE under the reducing condition. Phosphorylated and total IGF-1R proteins were detected by western blot analysis using anti-phospho-specific IGF-1R antibody (Invitrogen, Camarillo, CA, USA) and anti-IGF-1R antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. Serum IGF bioactivity can be evaluated by the phosphorylation levels of IGF-1R. As a result, IGF bioactivity in the patient's serum was greater than in a normal subject (Figure 3B, lanes 1 and 4). In addition, to clarify the ability of the anti-IGF neutralizing antibodies to inhibit the patient's IGF bioactivity *in vitro*, N7-IGF-1R cells was stimulated by the diluted serum sample (1mg protein) with anti-IGF-2 specific antibody (ab9574) and anti-IGF-1 and IGF-2 neutralizing antibody (KM1468; rat IgG2b), and bioactivity was evaluated by the same procedure of protein gel blot analysis as described above. The detailed characterization of KM1468 has been published elsewhere.[11] Increased IGF bioactivity in the patient's serum was inhibited by the anti-IGF-2 specific neutralizing antibody, ab9574 (Figure 3B, lane 2) to almost normal level of IGF bioactivity (Figure 3B, lane 4). Furthermore, Increased IGF bioactivity in the patient's serum was completely inhibited

by KM1468 (Figure 3B, lane 3), whereas in a normal serum no difference of inhibitory effect was observed between the anti-IGF-2 specific antibody and KM1468 (Figure 3B, lanes 5 and 6). These results demonstrated that not only bioactive IGF-2 but also bioactive IGF-1 were increased in the NICTH patient's serum.

The mainstay of treatment is surgical resection,[3–5] but in the present case the primary and metastatic lesions included large masses with rapid progression. Thus, systemic chemotherapy with cisplatin and etoposide was initiated, and the patient achieved a stable condition after the first five cycles. Furthermore, to relieve hypoglycemia, therapies recommended by previous studies, such as glucagon, recombinant human growth hormone, or high-dose glucocorticoids, were not used because the patient's blood glucose levels were able to be maintained with the administration of glucose via a central venous line.[3–5] Despite systemic chemotherapy being repeated, the patient succumbed to cancer progression 8 months after diagnosis.

Discussion

NICTH can cause an emergent and life-threatening condition, and should be suspected in any patient suffering from various solid tumors with hypoglycemia without a clear etiology.[3–6] In addition, NICTH is considered to cause hypoglycemia by increasing the insulin-like bioactivity of the circulating IGF system.[3–9] In the present case, we confirmed for the first time that the levels of circulating bioactive IGFs, mainly bioactive IGF-2, were highly elevated in the NICTH patient serum by using the modified KIRA.

Highly decreased levels of total IGF-1, insulin, and C-peptide are typical laboratory data for NICTH.[3–6] Because it has been demonstrated in many reports that HMW IGF-2 is secreted by the tumor itself and disappeared with relief from the hypoglycemic symptoms after successful removal of the tumor, this characteristic peptide in the patient circulation has been thought to play an important role in activation of the IGF system in situations without elevation of total IGF-2.[3–9]

HMW IGF-2 severely compromises IGFBP-3-based ternary complex formation, whereas all IGFBPs are able to form both mature and HMW IGF-2.[8,12–14] Consequently, a shift to binary complexes is likely to occur.[8,12–14] Binary complexes are thought to enable paracellular penetration of the vascular barriers to diffuse more effectively in the tissue microenvironment.[8,12,13] In addition, the short half-life and high turnover rate of binary complexes lead to increased delivery of bioactive IGFs to tissue receptors.[8,12,13,15] Moreover, it has been reported in recent studies that HMW IGF-2 exhibits a similar degree of bioactivity to both IGF-1R and IR as mature IGF-2.[16] Even in situations with an increase of HMW IGF-2 in the circulation, free IGFs in bioactive forms still need to be released from the IGF–IGFBP complex. In fact, Frystyk *et al*. demonstrated that the

levels of free IGFs were highly elevated in the patient serum.[9] On the other hand, some reports are available about NICTH cases involving only a small increase of HMW IGF-2.[8,9] Although the mechanism responsible for the increase of free IGFs has not yet been fully elucidated, free IGFs have been strongly suggested as being of major importance in provoking hypoglycemia in NICTH patients.[3,4,6,9,14]

IGF-2 binds to IR-A and -B with an affinity of 35–40% and about 5% that of insulin, respectively. However, the serum concentration of IGF-2 is about 100–1000 times higher than that of insulin.[4,17] In theory, in patients with NICTH, increased IGF bioactivity on the IR may fully induce hypoglycemia.[3,4,7–9] The mechanism of hypoglycemia is attributed not only to enhanced glucose uptake by the tumor itself, but also to glucose consumption in the peripheral muscles and inhibition of glucose production in the liver through the IR (because no IGF-1R is expressed by the adult liver).[18]

In the present case, western blot analysis showed that HMW IGF-2 was detected faintly and total IGF-2 was lower than in the control serum. However, the levels of bioactive IGFs (IGF-1 and IGF-2) were obviously elevated in the patient; thus, bioactive IGFs could be considered to play a major pathogenic role in enhanced hypoglycemic insulin-like activity. Bioactive IGF-2 was thought to be the main etiological peptide because increased IGF bioactivity in the patient serum was inhibited to almost normal IGF bioactivity level by the anti-IGF-2 specific neutralizing antibody *in vitro*. In addition, immunohistochemical analysis demonstrated that the IGF signal was activated in this tumor, suggesting that tumor growth, at least in part, is dependent on the IGF pathway in an autocrine and/or paracrine manner, in agreement with previous reports.[3,4]

Furthermore, we have previously reported on the therapeutic efficacy of the anti-IGF-1 and IGF-2 neutralizing antibody, KM1468, in treating various tumors such as liver metastasis of colorectal cancers, bone metastasis of prostate cancer and breast cancer, and multiple myeloma *in vivo*.[11,19–21] In the present study, KM1468 completely inhibited increased IGF bioactivity *in vitro*. Frystyk *et al*. demonstrated that not only the levels of free IGF-2 but also free IGF-1 were highly elevated in the patient serum, and levels of free IGF-1 were fourfold increased in patients compared to that in normal subjects.[9] Therefore, both free IGF-1 and IGF-2 may contribute to the progression of the hypoglycemia.

Our finding suggested that by direct neutralization of bioactive IGFs, a tumor growth signal and insulin-like signal mediated by both IGF-1R and IR can be blocked simultaneously. Therefore, neutralization of bioactive IGFs might become a novel effective therapeutic strategy for NICTH to relieve the hypoglycemic symptoms together with the tumor suppressive effect.

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Conflict of interest

The authors declare that they have no conflict of interests.

Consent

Written informed consent was obtained from the patient for publication of this Case report and any accompanying images.

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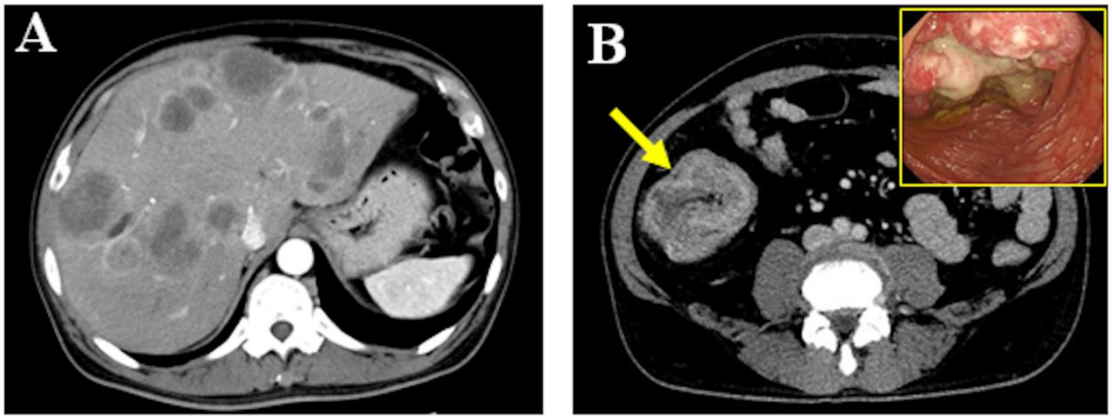

Figure 1. Radiological findings of this case.

(A) Abdominal computed tomography revealed multiple metastatic tumors in the liver and (B) thickening of the ascending colonic wall. (B, inset) Total colonoscopy demonstrated a large ulcerated and circumferential tumor at the same region.

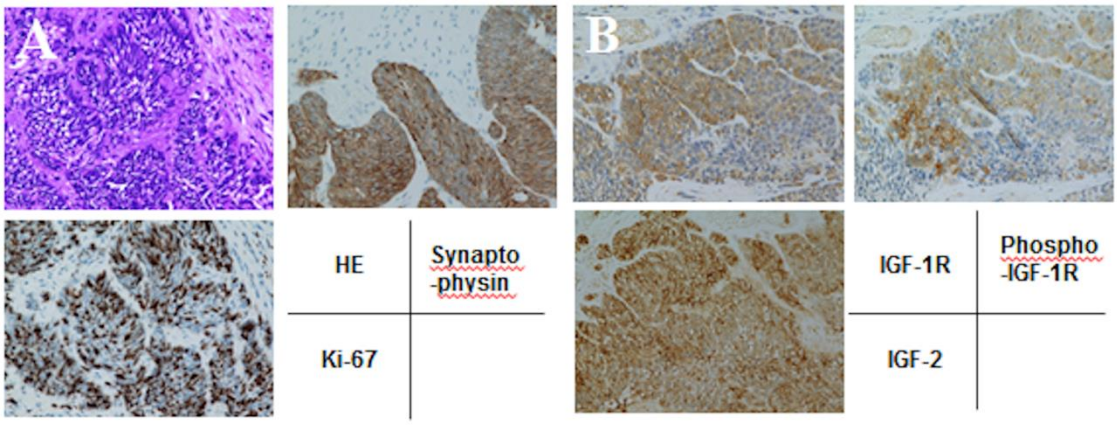

Figure 2. Immunohistochemical analysis of tumor cells.

(A) The tumor cells were highly atypical small cells with hyperchromatic nuclei and scanty cytoplasm, and strongly positive for synaptophysin and Ki-67 (labeling index; 70%) (×400). (B) The tumor cells were positive for IGF-2, IGF-1R, and phosphorylated IGF-1R (×400).

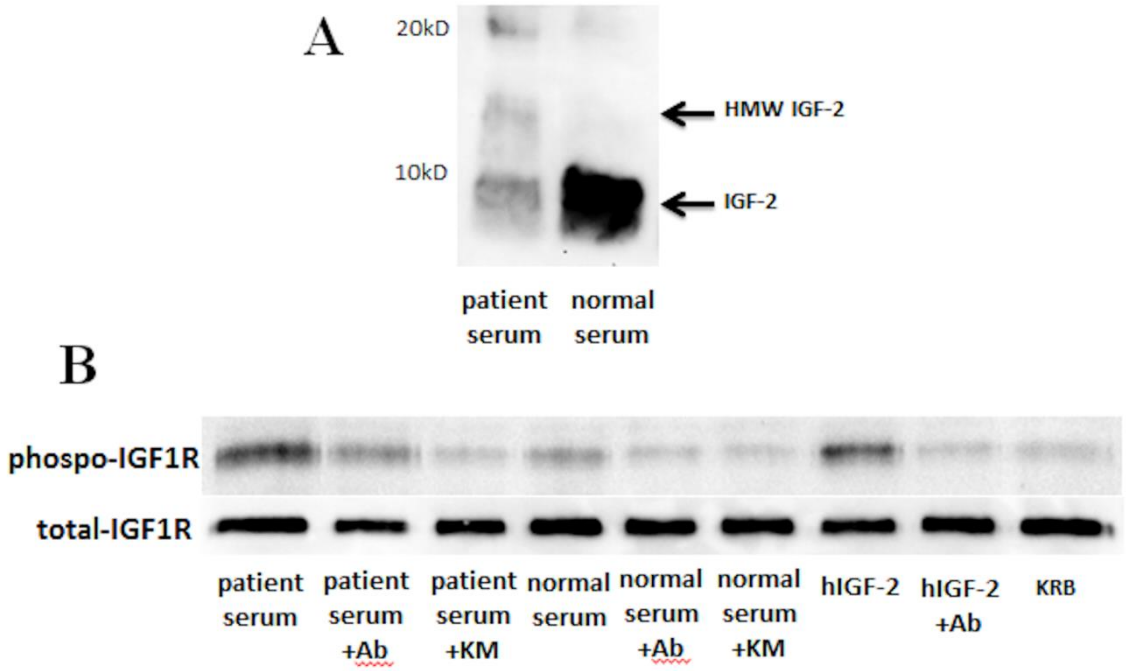

Figure 3. Western blot analysis and the kinase receptor activation assay of the patient's serum.

(A) High molecular weight (HMW) and mature IGF-2 were detected in serum by western blot analysis using anti-IGF-2 antibody (ab9574). Each serum (40 μg of protein per lane) was separated by 15% SDS-PAGE. (B) IGF bioactivity in the patient's serum was greater than in a normal subject, and increased IGF bioactivity was inhibited to normal bioactivity level by the anti-IGF-2 specific antibody, ab9574, and increased IGF bioactivity was completely inhibited by the anti-IGF neutralizing antibody, KM1468. KRB; Krebs–Ringer bicarbonate buffer as a negative control, Ab; ab9574 (anti-IGF-2 specific antibody, final concentration 10 μg/ml), KM; KM1468 (anti-IGF neutralizing antibody, final concentration 10 μg/ml), hIGF-2; recombinant human IGF-2 (final concentration 10 ng/ml) as a positive control.

Table 1. Laboratory data on admission

WBC	25.3×10^9	/L	($2.7–10.7\times10^9$)	Glu	24	mg/dL	(65–105)
RBC	4.59×10^{12}	/L	($3.62–5.36\times10^{12}$)	HbA1c	5.3	%	(4.6–6.2)
Plt	734×10^9	/L	($109–384\times10^9$)	Insulin Ab	<0.4	% (NSB)	(<0.4)
AST	42	IU/L	(12–30)	Insulin	0.6	μU/ml	(3–15)
ALT	18	IU/L	(10–42)	CPR	0.57	ng/ml	(1.0–2.5)
LDH	294	IU/L	(124–226)	GH	0.20	ng/ml	(<2.1)
ALP	867	IU/L	(115–359)	IGF-1	30	ng/ml	(59–215)
γ-GTP	655	IU/L	(9–54)	IGF-2	499	ng/ml	(396–1049)
T-Bil	0.3	mg/dL	(0.3–1.3)	IGFBP-2	596	ng/ml	(140–715)
T-Chol	130	mg/dL	(140–220)	IGFBP-3	1811	ng/ml	(1960–4650)
BUN	6	mg/dL	(8–22)	CEA	8.8	ng/ml	(<5.0)
Cre	0.58	mg/dL	(0.6–1.06)	CA19-9	71.1	U/ml	(<37)
CRP	4.6	mg/dL	(<0.2)	NSE	180.20	ng/ml	(<12.0)

The levels of total IGF-2, IGFBP-2, IGFBP-3 were measured using a commercially available ELISA kit (BioVendor, Karasek, Brno, Czech Republic).

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