Clinical implications of insulin-like growth factors through the presence of their binding proteins and receptors expressed in gynecological cancers

S. Hirano, N. Ito, S. Takahashi, T. Tamaya

Department of Obstetrics and Gynecology, Gifu University School of Medicine, Gifu (Japan)

Summary

Since insulin-like growth factors (IGFs) are known to play critical roles in the development of cancers, we examined the expression of the mRNA and protein of IGF-binding protein (IGFBP) and cognate receptors to assess their possible involvement in gynecological malignancy. The specimens were obtained from 46 endometrial, 32 cervical, and 20 ovarian cancers, and 28 normal endometrium.

In endometrial cancers, IGFBP-1, -2, -3 and IGF-1 receptor (IGF-1R) mRNAs were detected in 8.7, 89.1, 95.6, and 91.3% of tumors, respectively, and the corresponding proteins in 54.3, 54.3, 95.6, and 91.3% of tumors, respectively. Clinical staging was significantly related to the expression of IGFBP-1 and -2 proteins.

In ovarian cancers, their mRNAs were detected in 10.0, 90.0, 95.0, and 100.0%, and proteins in 15.9, 50.0, 90.0, and 80.0%. In cervical cancers, their mRNAs were detected in 6.3, 90.6, 96.8, and 87.5%, and proteins in 44.4, 18.8, 84.4, and 87.5%. IGF-1R was highly expressed in all specimens.

The abnormally balanced co-expression of IGFBPs and high levels of IGF-R in gynecological cancers suggest that IGF signals might be involved in the growth of these tumors.

Key words: IGFBP, IGF-R; Endometrial cancer; Cervical cancer; Ovarian cancer; Clinical implication.

Introduction

Insulin-like growth factors (IGF) acting through membrane receptors are involved in the growth of various types of human cells [1-6]. The growth-stimulatory and -inhibitory potentials of IGFs are additionally modulated by soluble specific IGF-binding proteins (IGFBPs) existing in multiple forms in plasma and tissues [1, 7, 8]. The distribution of IGFs and their receptors in a wide variety of organs and tissues enables IGFs to exert endocrine, paracrine and autocrine effects on their cell proliferation and differentiation [9]. Since postmenopausal women with endometrial cancers have increased serum insulin levels, insulin and IGFs may play a mitogenic role in the development of such cancers [10-12].

IGF receptors (IGF-R) are present in endometrial cancers, and patients harboring these tumors have increased plasma IGF-1 levels [13, 14] and decreased plasma levels of IGFBP-1 [15], implying that IGF signaling may play a role in the development and growth of endometrial cancers.

IGFs are not produced by epithelial cells [13, 16, 17], yet IGFBPs are produced by granulosa-luteal cells and are detected in the normal ovary and uterus [18-23]. Ovarian cancers express IGF-1, IGF-1R and IGFBPs [8, 11, 24-27]. Members of IGF families are thought to contribute to malignant transformation in HPV-infected cervical epithelium [28].

The present study was conducted to assess the relationships between the local expression of IGFBPs and IGF-1R proteins/mRNAs and clinical/pathological features in order to evaluate the role of IGF signaling in the malignant potential of gynecological cancers.

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Materials and Methods

Materials

The following antibodies against IGF-BP and IGF-1R were purchased commercially: anti-IGFBP-1 (RDI, Flanders, NJ), and anti-IGFBP-2 (Santa Cruz, CA, USA), anti-IGFBP-3 (RDI, Flanders, NJ, USA), anti-IGF-1R (Santa Cruz, CA, USA). The messenger RNA (mRNA) purification kit and nitrocellulose membranes were products of Pharmacia-LKB (Uppsala, Sweden). Moloney murine leukemia reverse transcriptase and Taq polymerase were from Takara-Shuzo (Tokyo, Japan). Hybond-N^+ membranes, the ECL-direct labeling and detection system, and the sequencing kit were from Amersham (Amersham, UK). All other chemicals were of reagent grade.

Tissue collection

The present study was approved by the ethics committee of Gifu University and the informed consent was obtained from all of the subjects.

Gynecological tumors and normal tissues were surgically collected at the Department of Obstetrics and Gynecology, Gifu University School of Medicine from January 1996 to December 2002. Part of the tissues were frozen in liquid nitrogen and stored at –80°C until they were used and another portion was subjected to pathological examination.

Normal eutopic endometrial tissues were collected from 28 patients (age; 38±5) with uterine myoma. The endometrial phase was judged based on histological evaluation. A total of 46

endometrial cancers(endometrioid carcinoma; age, 55±6), 20 ovarian cancers (adenocarcinoma; age, 54±7) and 32 cervical cancers (squamous cell carcinoma; age, 50±7) were examined in the study.

The patients were free of endocrine or systemic diseases, and preoperative therapies. Before surgery, serum was collected from all patients to assess tumor markers (CA125, CA19-9, CA72-4) using commercial procedures. Clinical features such as BMI and age, and obstetrical history were evaluated.

Protein extraction

Proteins were extracted from the specimens using standard techniques [29]. In brief, one volume of Laemmli's sodium dodecyl sulfate (SDS) sample buffer [29] was added to five volumes of tissue and the mixture was homogenized at RT by brief sonication and immediately boiled for five minutes. The mixture was then centrifuged at 100,000 x g for ten minutes. The supernatant was stored at -80°C. Protein content was determined by the method of Lowry *et al.* [30]

Immunoblotting

The levels of IGFBPs and IGF-1R in the tissues were determined by immunoblotting of the extracts with the following antibodies: anti-IGFBP-1 (RDI, Flanders, NJ), anti-IGFBP-2 (Santa Cruz, CA), anti-IGF-BP3 (RDI, Flanders, NJ), and anti-IGF-1R (Santa Cruz, CA), as previously described [8, 14, 24]. The solubilized protein was resolved by 10% polyacrylamid gel electrophoresis (PAGE). The proteins separated by electrophoresis were transferred to a nitrocellulose membrane and then probed with antibodies (1:3000). Biotin-labelled anti-rabbit whole antibody from donkeys (Amersham, Boston, MA) was used as a second antibody for Western blots.

RNA extraction and reverse transcription-polymerase chain reaction amplification

Total RNA was extracted from tissues using the guanidine isothiocyanate/acid phenol method [31]. A random primed complementary DNA (cDNA) library was made from 5 µg total RNA using Moloney murine leukemia reverse transcriptase under the conditions recommended by the supplier. The reaction product was heat-denatured at 95°C for 5 min, and quickly chilled on ice. The cDNA (1 µl) was amplified in a 50 µl reaction volume containing 100 mmol/l Tris-HCl (pH 8.3), 500 mmol/l KCl, 15 mmol/l $MgCl_2$, 2.5 mmol/l each deoxynucleotide triphosphate, 5 U/µl Taq polymerase, and 0.5 µmol/l primers. The sequences of oligonucleotide primers, designed according to the published sequences of human IGFBPs and IGF-1R [10, 32] were as follows: IGFBP-1, primer a (sense): 5'-AGGCTCTCCATGTCACCAAC-3'; primer b (antisense): 5'-TACATATATATATAAATGTG-3'. IGFBP-2, Primer c (sense): 5'-GGGCAAGGGTGGCAAGCATC-3'; primer d (antisense): 5'-AATCCTCCAGCACCCACCAC-3'. IGFBP-3, primer e (sense): 5'-CTGCGTCAACGCTAGTGCCG-3'; primer f (antisense): 5'-TGGGACTCAGCACATTGAGG-3'. IGF-1R, primer g (sense): 5'-AGCTAAACCGGCTAAACCCG-3'; primer h (antisense): 5'-ACCACACCCTTGGCAACTCC-3'. Each tube contained a set of sense and antisense primers giving rise to a predicted DNA fragment of 385 bp (a and b), 450 bp (c and d), 358 bp (e and f), or 390 bp (g and h). In the case of receptor mRNA, the primer set was designed to flank the entire transmembrane domain. Thirty-five cycles of amplification were carried out , with each cycle consisting of denaturation at 90°C for 45 sec, annealing at 55°C for 45 sec, and extension at 72°C for 90 sec followed by a final extension for 5 min at 72°C. The DNA product (10 µl) was electrophoresed on a 2% agarose gel, and bands were visualized using a UV transilluminator after ethidium bromide staining. Sequence of the PCR products was analyzed and confirmed manually by the dideoxy chain termination method [33] using Sequenase II according to the manufacturer's protocols.

Statistics

For the statistical analysis, χ^2 or Fisher's exact probability test was used. Differences were considered to be significant if p was less than 0.05.

Results

I. Detection of IGFBP mRNAs and proteins

I.1. Endometrial cancers and normal endometrium

In endometrial cancers (endometrioid carcinoma), IGFBP-1, -2 and -3 mRNAs were expressed in 8.7, 89.1, and 95.6% of tumors, respectively (Table 1 and Figure 1), while the protein positive rates were 54.3, 54.3, and 95.6%, respectively (Table 2 and Figure 2). The level of IGFBP-1 mRNA expression was extremely low, while that of IGFBP-2 was high. IGFBP-1 and -2 proteins were detected in approximately half of the endometrial cancers. The expression of IGFBP-3 was highly detected at both the mRNA and protein levels. In cases positive for IGFBP-2 and -3 protein, the respective mRNAs were detected at a high frequency (over 90%), yet in IGFBP-1 protein-positive cases, the mRNA was rarely detected (4/25).

In the endometrium as a normal counterpart tissue, however, IGFBP-1 expression was rarely detected at either the mRNA or protein level. IGFBP-2 and -3 mRNAs were frequently detected but the respective protein detection rates were relatively low (Tables 1 and 2).

Table 1. — *Expression rate (%) of IGFBP-1, -2, -3 and IGF1-R mRNAs in gynecologic cancers.*

	n	IGFBP-1	IGFBP-2	IGFBP-3	IGF-1R
Endometrial ca.	46	8.7	89.1	95.6	91.3
Ovarian ca.	20	10.0	90.0	95.0	100.0
Cervical ca.	32	6.3	90.6	96.8	87.5
Endometrium	28	7.1	92.9	89.3	96.4

ca., cancer.

Table 2. — *Detection rate (%) of IGFBP-1, -2, -3 and IGF1-R proteins in gynecologic cancers.*

	n	IGFBP-1	IGFBP-2	IGFBP-3	IGF-1R
Endometrial ca.	46	54.3	54.3	95.6	91.3
Ovarian ca.	20	15.9	50.0	90.0	80.0
Cervical ca.	32	44.4	18.8	84.4	87.5
Endometrium	28	8.9	8.9	35.7	85.7

ca., cancer.

Table 3. — *Relationship of positive IGFBP-1 protein detection and clinical stage in endometrial cancers.*

	IGFBP-1	
	Negative	Positive
Stage I-II	21	13
Stage III-IV	0	12

p = 0.00022.

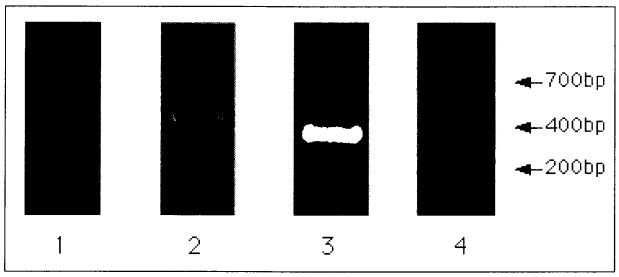

Figure 1. — Expression of mRNAs of IGFBPs, and IGF-1R in endometrial carcinoma.
IGFBP-1: lane 1. IGFBP-2: lane 2. IGFBP-3: lane 3. IGF-1R lane 4. The profile of a representative profile case is shown.

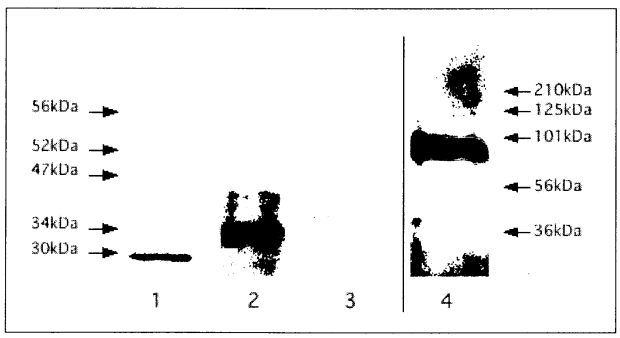

Figure 2. — Detection of IGFBPs and IGF-1R protein in endometrial carcinoma.
IGFBP-1: lane 1. IGFBP-2: lane 2. IGFBP-3: lane 3. IGF-1R lane 4. The profile of a representative profile case is shown.

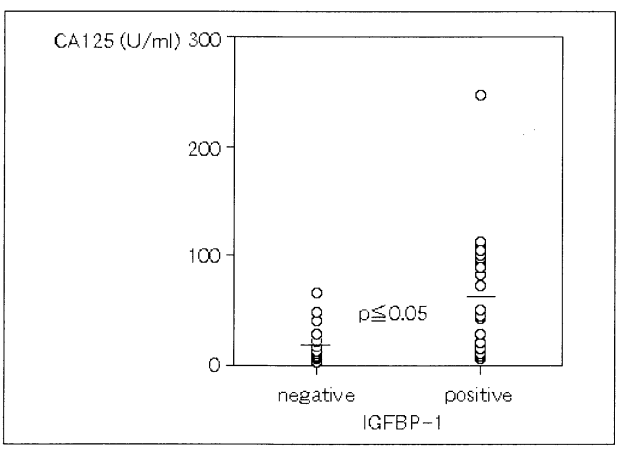

Figure 3. — Relationship of IGFBP-1 expression in cancers and plasma CA125 levels in endometrial cancer patients.

Regarding differences during the menstrual cycle, no significant difference of IGFBP detection rates appeared to exist between secretory- and proliferative-phase endometrium (data not shown).

Thus, IGFBP-1, -2, and -3 may be up-regulated in endometrial cancers or absorbed by them from the circulation, as compared with the normal emdometrial counterpart.

Clinical stage was inversely related to IGFBP-1 and -2 protein detection rates ($p < 0.02$) as shown in Tables 3 and 4, but not to that of IGFBP-3 (data not shown). There

Table 4. — *Relation of positive IGFBP-2 protein detection and clinical stage in endometrial cancers.*

	IGFBP-2	
	Negative	Positive
Stage I-II	19	15
Stage III-IV	2	10

$p = 0.0190$.

was a significant relationship between the plasma CA125 level and IGFBP-1 positivity ($p < 0.05$) as shown in Figure 3. BMI and various other tumor markers tested were not significantly related to the expression rate of IGFBPs (data not shown).

I.2. Ovarian cancers

In ovarian cancers (adenocarcinoma), IGFBP-1, -2 and -3 mRNAs were expressed in 10.0, 90.0, and 95.0% of tumors, respectively, and the corresponding proteins in 15.9, 50.0, and 90.0% of tumors, respectively, as shown in Tables 1 and 2. The detection rates of IGFBP-1 mRNA/protein were especially low and IGFBP-2 was down-regulated. Histological grade, clinical staging, BMI and tumor markers were not related to the expression rate of IGFBPs (data not shown).

I.3. Cervical cancers

In cervical cancer (squamous cell carcinoma), IGFBP-1, -2 and -3 mRNAs were expressed in 6.3, 90.6, and 96.8% of tumors and the corresponding proteins in 44.4, 18.8, and 84.4% of tumors, respectively, as shown in Tables 1 and 2. IGFBP-1 was up-regulated and IGFBP-2 was down-regulated. The clinical features noted above regarding ovarian cancers were not related to the expression rate of IGFBPs (data not shown).

II. Detection of IGF-1R mRNA and protein

Generally, IGF-1R mRNA was detected in a high proportion of endometrial cancers (91.3%), normal endometria (96.4), ovarian cancers (100.0) and cervical cancers (87.5) (Tables 1 and 2), which was in general consistent with the analysis of protein by immunoblotting. This evidence implies that IGF receptor signaling contributes to the growth of gynecologically normal and malignant tissues. As expected, BMI and other clinical features were not related to the rate of detection of IGF-1R.

Discussion

The physiological roles of IGFs and IGFBPs in the female genital tract have been elucidated in a number of previous studies after the initial observations of the existence of IGFs, IGF-R, and IGFBPs[5, 18-22, 34-36]. Little is known about the role of IGF signaling in gynecological cancers. Recent studies, however, have demonstrated the expression of IGF-1, IGF-R and IGFBPs in ovarian cancer cells [8, 11, 13, 24-27, 37-39] and endometrial cancers [5, 9, 11, 13-17], implying that IGF signaling might play a role in the endocrine/autocrine growth regulation of endometrial and ovarian cancers. The present study showed that the balance of IGFBPs

was abnormal and IGF-R was highly expressed in cancers originating from the genital tract. The presence of these factors was consistent with the previously reported data mentioned above. The present study extended the previous observations in several aspects, in particular, by examining the relationships of the expression of IGF receptor and IGFBPs and clinical features.

IGF-R was expressed even in IGFBP-negative specimens. Plasma IGF levels are increased in endometrial cancers even when samples are matched for BMI of the patients [6, 15], suggesting that IGFs might be involved in endometrial cancer risk. The present data showing the frequent expression of IGF receptors in endometrial cancers and the high rate of positivity of IGFBP-1 and -2 in advanced stage endometrial cancers suggest that IGF siganaling might contribute to the malignant potential of endometrial cancers.

IGFBP-1, -2, and -3 were up-regulated in the endometrial cancers compared with the normal counterpart endometrium, suggesting that IGFBPs may be up-regulated locally or absorbed from the circulation, and that it may be important in endometrial cancer progression.

In endometrial cancer patients, the plasma CA125 level is related to the clinical progression [40] and, in the present study, it was increased and related to the IGFBP-1 protein positivity of the cancer tissues, suggesting that local IGFBP-1 protein level may be related to endometrial cancer progression.

Taking previous data [8, 11, 13, 24-27, 37-39] on ovarian cancers into consideration, the present investigation suggests that the abnormal balance of IGFBPs and the high rate of IGF-R expression may be related to constitutive IGF signaling in ovarial cancers and unrelated to clinical malignancy.

In cervical cancers, as in ovarian cancers, there was no relationship between expression/detection rates of IGFBP, IGFBP balance, high IGF-R expression and clinical features. IGF signaling is constitutively present in cervical cancers.

Conclusion

Gynecological cancers express an abnormal balance of IGFBPs and a high level of IGF-R, implying the presence of IGF signaling, which gives malignant potential to these cancers especially to endometrial cancers. In general, the expression of IGFBPs and IGF-R suggests signaling by these factors in an endocrine, paracrine or autocrine fashion, and their possible roles in the malignant potential of gynecological cancers.

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Address reprint requests to:
N. ITO, M.D.
Department of Obstetrics and Gynecology
Gifu University School of Medicine
Gifu, Tsukasamachi 40
Gifu 500-8705 (Japan)