Serum Insulin-like Growth Factors (IGFs) and IGF Binding Protein (IGFBP)-3 in Patients with Gastric Cancer : IGFBP-3 protease activity induced by surgery

Recent findings have indicated that insulin-like growth factors (IGF-I and IGF-II) may play a role in neoplasia. Alteration of serum IGFs or IGF Binding Proteins (IGFBPs) have been reported in some tumors. In this study, we measured serum IGF-I, IGF-II and IGFBPs profile in gastric cancer by radioimmunoassay and Western ligand blots. The serum IGF-I level in gastric cancer was significantly lower than in control subjects (65.2±26.5 vs 148.4±55.2 ng/ml, p< 0.01) and was further decreased to 45.5±20.9 ng/ml after surgery. The serum IGF-II level was slightly higher than that in control subjects (826.3±360.2 vs 735.7±154.6 ng/ml) but it was significantly decreased after surgery (525.7± 220.1 ng/ml, p<0.05). The serum IGFBP-3 level was not significantly different from those in control subjects. However, we observed a decreased level of serum IGFBP-3 after surgery, and incubation of postoperative serum with control serum resulted in a significant reduction of IGFBP-3 level. The reduction of IGFBP-3 in postoperative serum was mainly due to surgery associated IGFBP-3 protease activity. This protease activity was totally inhibited by aprotinin, EDTA and PMSF but not by pepstatin and leupeptin. This inhibition pattern is consistant with cation dependent serine protease. We speculate that proteolysis of IGFBP-3 may contribute to increase the bioavailability of IGFs. ***(JKMS 1997; 12 : 32~9)***

Key Words : *Insulin-like growth factors(IGFs), IGF binding protein-3, IGFBP-3 protease activity, Gastric cancer*

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INTRODUCTION

Insulin-like growth factors, IGF-I and IGF-II, are important mitogenic and anabolic peptides both in vivo and in vitro, and are thought to be significant endocrine and autocrine-paracrine factors involved in normal and malignant cellular proliferation (1~3). A family of at least six proteins with a high affinity for the IGFs, IGF Binding Proteins (IGFBPs), distinct from the IGF receptors, has been identified and are believed to modulate the proliferative and mitogenic effects of IGFs on cells (4, 5). In the serum of normal adults, most IGF is bound in a 150 kDa complex composed of IGF-I or IGF-II, acid-labile subunit (ALS) and IGFBP-3 (6). An additional complexity in the regulation of IGFs by IGFBPs has recently reported IGFBP proteases (7~9). The IGFBP-3 proteases degrade IGFBP-3 into smaller fragments, which may have reduced affinity for IGFs (10). Alteration of IGFBP-3 functionality or structure by protease is hypothesized to alter the release of IGFs from IGFBPs and may be critical in the regulation of IGF bioactivity and bioavailability. In humans, these protease activities have been found during pregnancy serum (8), and in patients suffering from severe catabolism (11).

A wide variety of human tumors have been characterized as expressing an unusually large amount of IGF-I or -II mRNA or protein (12, 13). Virtually all cells that are known to secrete IGFs also secrete IGFBPs. Several breast cancer cell lines secrete IGF-I and also IGFBPs (14, 15). Daughaday (16) reported that many mesenchymal tumors have increased synthesis of proIGF-II and low serum IGF-I level. Cohen et al.(17) reported the elevation of serum IGFBP-2 in patient with prostate cancer.

The goals of this study were to characterize the serum IGFs and IGFBP-3 in gastric cancer and to determine if IGFs and IGFBP-3 change after surgery, another catabolic state.

MATERIALS AND METHODS

Subjects

A total of 20 adult patients with primary gastric adenocarcinoma were enrolled in this study. A clinical description of the subjects and cancer stage is provided

Table 1. Patients characteristics and stages

Stages	No of cases	Age(year) Mean±SD	M : F ratio
Stage I	7	47.7±10.3	5 : 2
Stage II	5	54.2±13.2	5 : 0
Stage III	6	46.3± 9.6	4 : 2
Stage IV	2	56.5± 0.7	2 : 0
Total	20	49.8±10.9	16 : 4

in Table 1. There were 16 males and 4 females, with ages ranging from 30 to 68 years. Patients were excluded if they had hypothyroidism, renal insufficiency (serum creatinine>2 mg/dl), liver dysfunction (serum aspartate aminotransferase more than twice normal) or severe malnutrition (serum albumin<3 g/dl). Cancer staging was performed according to the TNM staging groups approved by UICC and AJC(18). The patients took nothing by mouth for 12 hours before surgery and they usually took food normally after 7 days of surgery. This study was approved by the Ethics Committee of the Chonbuk National University Hospital.

Samples

Blood samples were obtained before and 1 day after surgery from 20 patients with gastric cancer. From 5 of 20 patients, blood samples were collected before and after 1, 2, 7 and 10 days of surgery. Aliquots of sera were stored at -70℃ until used. We also obtained blood samples from 20 age, sex-matched healthy adults. To compare the effects of surgery with those of fasting alone on IGFBPs, blood samples were collected from 4 healthy adults before fasting and after 2 days of fasting.

Reagents

Peptide and anti-serum : Recombinant human IGF-I was purchased from Bachem (Torance, CA, USA). IGF-I was iodinated by a modification of the chloramine T method to a specific activity of 150-300 uCi/ug. Polyclonal anti-IGF-I anti-serum, a generous gift of Drs. LE Underwood and JJ Van Wyk (University of North Carolina at Chapel Hill) was distributed through the Hormone Distribution Program of NIDDK to National Hormone and Pituitary Program.

Protease inhibitors : Aprotinin, PMSF, EDTA, leupeptin and pepstatin were purchased from Sigma Chimical Co. (St. louis, MO, USA).

IGF-I Radioimmunoassay (RIA)

To seperate IGF peptides from their binding proteins, 500 ul of serum was chromatographed in 0.1% formic acid on a 1.0×100 cm column containing Sephadex G-50 fine. The fractions eluting between 50 and 70 ml, which containing 90% of the IGF peptide activity, were collected in a glass tube containing 1.0 ml of 1% BSA, lyophilized and reconstituted 1 ml in RIA buffer. Serum IGF-I concentration was determined by RIA using ^{125}I-IGF-I and polyclonal anti-somatomedin-C antiserum. The inter- and intra-assay coefficients of variation were 7% and 5%, respectively, for the IGF-I RIA.

IGF-II Immunoradiometric assay (IRMA)

For the serum IGF-II, we used extracted sample by acid column chromatography, and measured the serum IGF-II level by IGF-II IRMA assay kit (Diagnostic System Laboratories Inc., Webster, Texas, USA).

Free IGF-I IRMA

For the free serum IGF-I, we used unextracted saples, and measured free IGF-I level by free IGF-I IRMA assay kit (Diagnostic System Laboratories Inc., Webster, Texas, USA)

Western ligand blot (WLB)

Three ul of serum or 100 ul of fractionated serum were electrophoresed on 10% SDS-PAGE under non-reducing condition with molecular weight marker (Biorad, Richmond, CA, USA). Electrophoresed proteins were electroblotted onto nitrocellulose, incubated with 2×10^6 cpm of ^{125}I-IGF-I and exposed to x-ray films for 5-7 days according to the method of Hossenlopp et al. (19).

Neutral size-exclusion chromatography

To determine the profile of IGF-I binding to IGFBPs, 500 ul of pooled serum obtained from 10 patients before and after 1 day of surgery were incubated with 50,000 cpm of ^{125}I-IGF-I for 18 hours at 4℃. The mixture was applied to a Sephacryl S-200 column (1.0×100 cm) equilibrated in 0.05 M sodium phosphate buffer, pH 7.4, and calibrated with r-globulin (158 kDa), hemoglobin (65 kDa), ovalbumin (44 kDa), cytochrome C (14 kDa) and iodinated IGF-I (7.5 kDa). The sample was eluted with 0.05 M sodium phosphate buffer at a flow rate of 15 ml/hour. Fractions of 1.5 ml were collected and assessed for radioactivity. Additionally, WLB was completed on individual fraction of serum.

IGFBP-3 protease assay

IGFBP-3 protease assays were performed by the method of Lamson et al.(20), with minor modification. Equal volume of pooled healthy control serum and test sera from 10 patients were mixed together and diluted in 50 ul of 0.5 mM $CaCl_2$. These mixtures were incubated for 6 hours at 37℃. Protease inhibitors were added

Table 2. Serum IGF-I and -II concentration in gastric cancer

	Gastric cancer		Control subjects
	preoperative (n=20)	postoperative (n=20)	(n=20)
IGF-I (ng/ml)			
total	65.2 ±26.5*	45.5 ±20.9**	148.4 ±55.2
free	1.05± 0.45	2.21± 0.96##	1.13± 0.42
free/total(%)	1.67± 0.59#	3.03± 0.84##	0.76± 0.26
IGF-II (ng/ml)	826.3±360.2	525.7±220.1##	735.7±154.6

Values are mean±standard deviation,
*$p<0.01$ compared with control value,
**$p<0.01$ compared with preoperative value,
#$p<0.05$ compared with control value,
##$p<0.05$ compared with preoperative value

as indicated and the digests were electrophoresed on 10% SDS-PAGE under non-reducing condition and analysed by WLB.

Statistics

Values are expressed as mean ± standard deviation. Student's t-test was used for statistical analyses of serum IGF-I and -II values.

RESULTS

The concentration of serum IGF-I in gastric cancer, as determined by RIA, was significantly lower (65.2±26.5 ng/ml, $p<0.01$, table 2) compared to that in control subjects (148.4±55.2 ng/ml). The decreased serum IGF-I level was further decreased after surgery (45.5±20.9 vs 65.2±26.5 ng/ml, $p<0.01$). The serum free IGF-I concentration was not elevated in gastric cancer compared to that in controls (1.05±0.45 vs 1.13±0.42 ng/ml), but after surgery, it was significantly increased to 2.21±0.96 ng/ml. In contrast, serum IGF-II level was slightly higher than that in control subject (826.3±360.2 vs 735.7±154.6 ng/ml, $p>0.05$, table 2), but serum IGF-II was significantly decreased after surgery (525.7±220.1 vs 826.3±360.2 ng/ml, $p<0.05$). However, the degree of serum IGF-I and -II changes did not correlate with gastric cancer stages (data not shown).

A Western ligand blot of sera from 15 patients with gastric cancer and control subject is provided in Figure 1. Pooled control serum (lane 1) had two major bands, corresponding to the glycosylated form of IGFBP-3, with molecular weight (MW) ranging between 37-43 kDa. Another faint band was seen in the control serum with MW of 24 kDa, corresponding to IGFBP-4. As can be seen in Figure 1, serum IGFBP patterns in gastric cancer were not significantly different from those in control subjects. The principal serum IGFBP in gastric cancer was IGFBP-3. Even though 4 of 15 patients showed slightly decreased serum IGFBP-3 level (lanes 4, 6, 12 and 16), mean serum IGFBP-3 level was similar to control level (16.01±5.45 vs 17.09±1.1 AUD, arbitrary densitometry unit, $p>0.05$). However, serum IGFBP-3 was dramatically decreased after surgery (Fig. 2, lanes 3, 5, 7, 9, 11 and 13). The decreased levels of serum IGFBP-3 were returning toward normal 7 days after surgery (Fig. 3, lanes 4 and 7) but they didn't return to normal until 10 days after surgery (lanes 5 and 8). In contrast, in fasted healthy subjects who did not undergo surgery, serum IGFBP-3 levels did not significantly decrease after 2 days of fasting (Fig. 4, lanes 2 and 4).

To determine the profile of IGF-I to IGFBPs, pre and postoperative serum samples from 10 patients were incubated with ^{125}I-IGF-I overnight at 4℃ and subjected

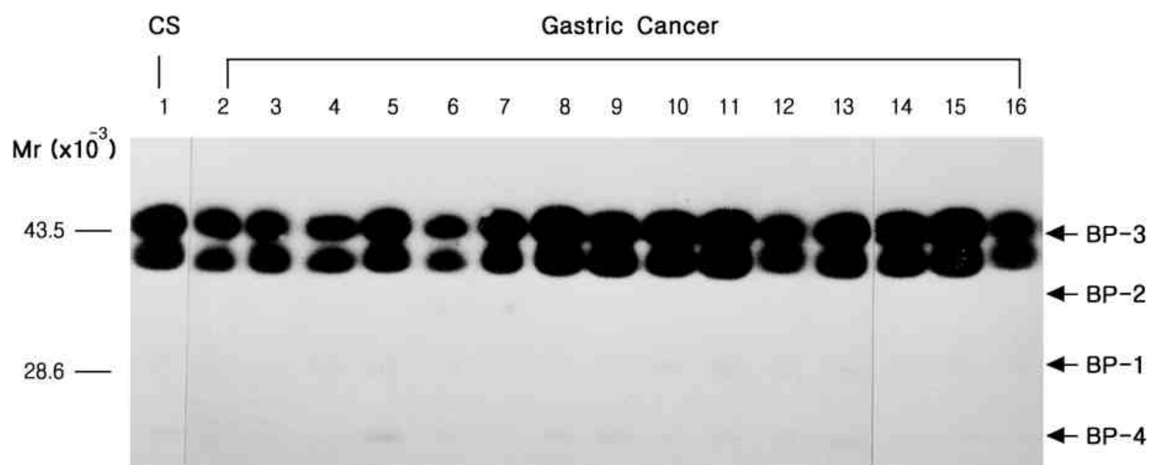

Fig. 1. *Serum IGFBP pattern in fifteen gastric cancer patients by WLB.* Lane 1, pooled control subject(CS) ; lanes 2-16, individual cancer patient. Arrows indicate IGFBP-1, -2, -3 and -4. Standard molecular weight is expressed in kilodaltons.

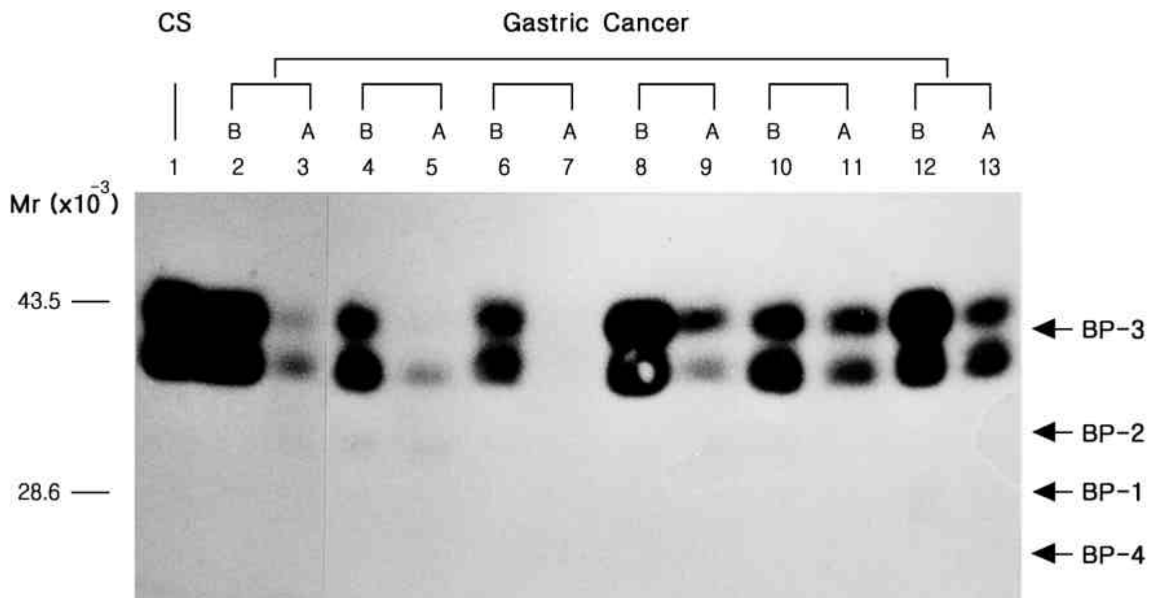

Fig. 2. *Changes in serum IGFBP-3 before and after surgery.* Sera were obtained before(B) and after(A) surgery from six gastric cancer patients. Lane 1, pooled control subject(CS) ; lanes 2-13, individual cancer patient. Arrows indicate IGFBP-1, -2, -3 and -4.

to neutral size-exclusion chromatography. Each fraction was counted for radioactivity and then WLB was performed. Since all patients showed a similar patterns, a representative chromatogram was shown. As illustrated in Figure 5A, preoperativie serum showed three peaks and radiolabled IGF-I was incoperated into 150 kDa complex more than 50 kDa complex (upper panel). The 150 kDa complex contained a large amount of IGFBP-3 dimer (lower panel, lanes 2-6), whereas the 50 kDa complex contained a small amount of IGFBP-3, -2, -1 and -4 (lanes 7-12). Thus, most of the IGFBP-3 exist as part of the 150 kDa ternary complex in preoperative serum. However, in postoperative serum, the amount of the total recovered radiolabled IGF-I associated with 150 kDa region was significantly lower than that in preoperative serum (Fig. 5B, upper panel). The 150 kDa complex contained only a small amount of IGFBP-3 dimer

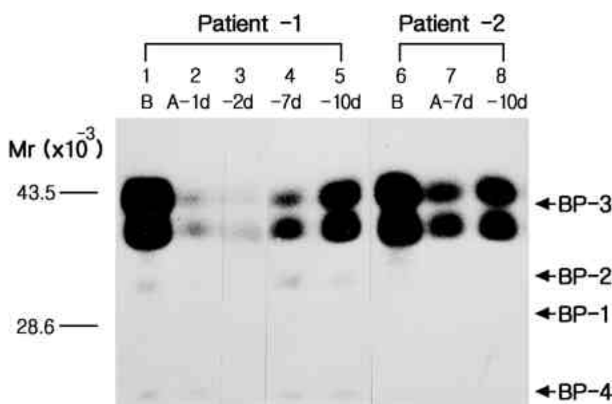

Fig. 3. *Changes in serum IGFBP-3 after surgery.* Sera were obtained before(B) and after(A) 1, 2, 7 and 10 days of surgery in patient 1 (lanes 1-5), and before(B) and after(A) 7 and 10 days of surgery in patients 2 (lanes 6-8). Arrows indicate IGFBP-1, -2, -3 and -4.

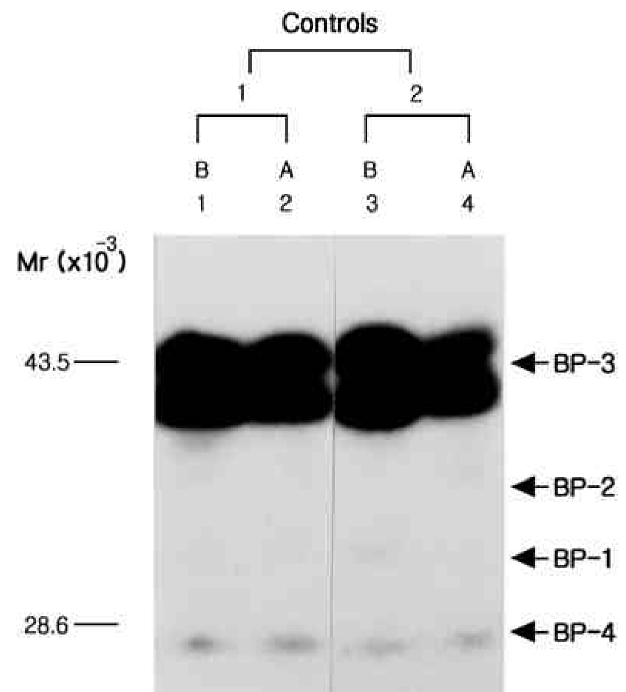

Fig. 4. *Changes in serum IGFBP-3 after fasting.* Sera were obtained before(B) and after(A) 2 days of fasting in 2 healthy control subjects. Arrows indicate IGFBP-1, -2, -3 and -4.

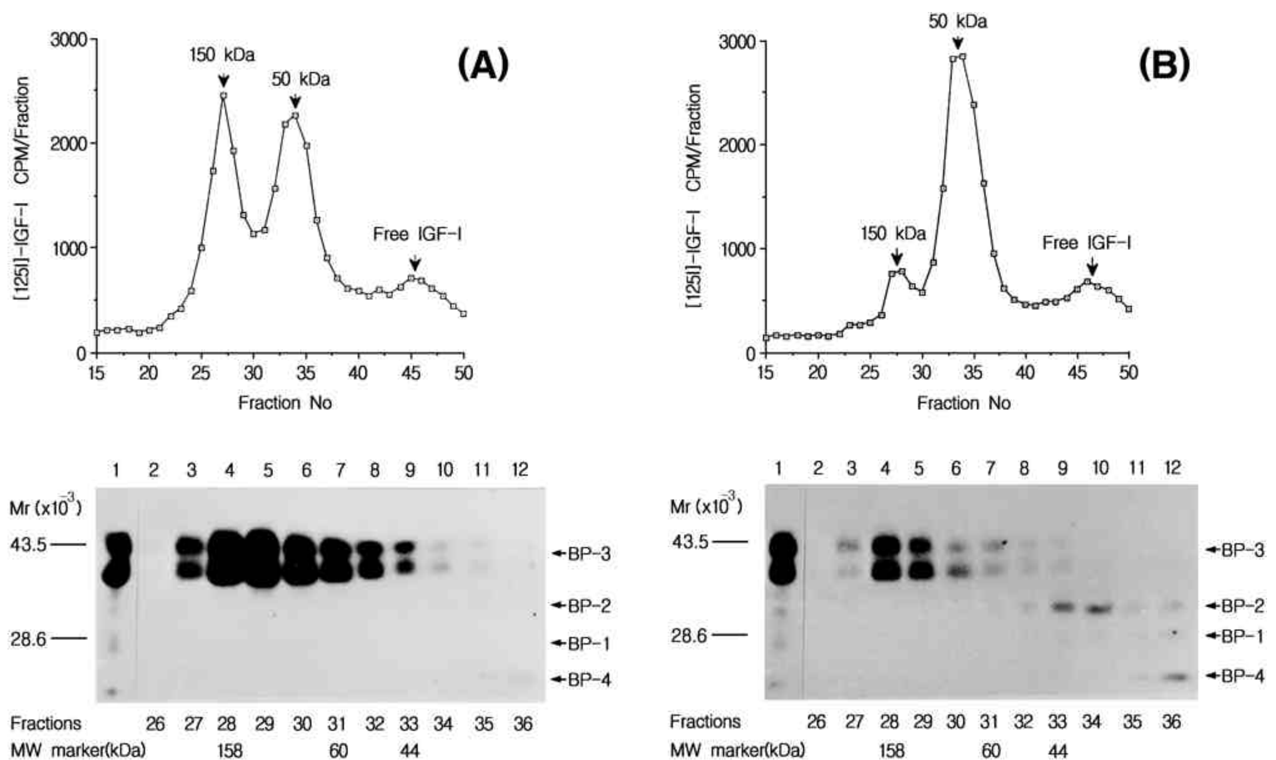

Fig. 5. *Characterization of IGFBP regions after size exclusion chromatography combined with WLB.* Upper panel, the pattern of radioactivity eluting from a Sephacryl S-200 column after incubation of ^{125}I-IGF-I with preoperative(A) and postperative serum(B). 150 and 50 kDa represent the eluting peak for the 150 and 50 kDa complexes, respectively. Third peak shows unbound ^{125}I-IGF-I. Lower panel, WLB analysis of fractionated serum(100 ul/lane) through the Sephacryl S-200 column. Lane 1 contains 3 ul of control serum. Lanes 2-6 represent 150 kDa IGFBPs complex, and lanes 7-12 represent 50 kDa IGFBPs complex. Arrows indicate IGFBP-1, -2, -3 and -4.

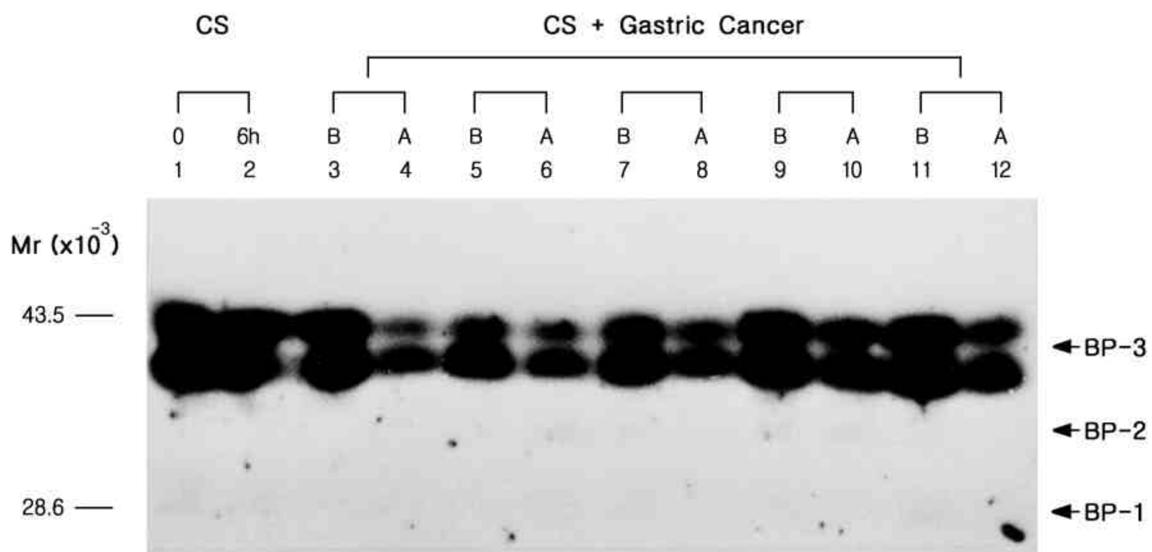

Fig. 6. *Proteolysis of IGFBP-3 by postoperative serum.* Sera were obtained before(B) and after(A) from five gastric cancer patients. Equal volumes of test sera and control serum were incubated for 6 hours. Then, aliquots of each sample were electrophoresed, analysed by WLB. Control surum alone(0 h and 6 h incubation) is shown in lane 1 and 2. Sera from five patients are shown in lanes 3-12. Arrows indicate IGFBP-1, -2, -3 and -4.

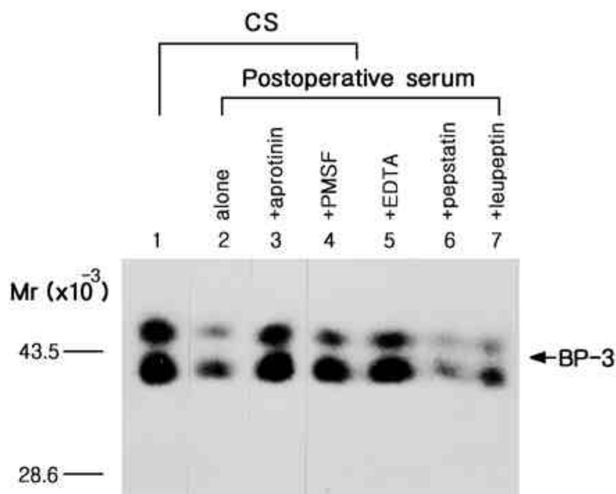

Fig. 7. *Effects of enzyme inhibitors on surgery induced IGFBP-3 protese activity.* Control serum was incubated for 6 hours at 37℃ with buffer alone(lane 1), or with postoperative serum(lane 2), or with postoperative serum+aprotinin, 25 uM(lane 3), +PMSF, 25 mM(lane 4), +EDTA, 5 mM(lane 5), +pepstatin, 25 uM(lane 6), +leupeptin, 25 uM(lane 7). The mixtures were electrophoresed, analysed by WLB. Arrow indicates IGFBP-3.

compared to preoperative serum (lanes 2-6). In contrast, 50 kDa complex containing IGFPB-3, -2, -1 and -4 was increased.

To investigate whether the proteolysis of IGFBP-3 explains the marked reduction of serum IGFBP-3 after surgery, pre and postoperative serum samples from 5 patients were mixed with pooled control serum and then incubated for 6 hours at 37℃. As can be seen in Figure 6, despite incubation with control serum or preoperative serum for 6 hours, glycosylated IGFBP-3 were not reduced (lanes 2, 3, 5, 7, 9 and 11). However, the marked reduction of IGFBP-3 bands were observed in the postoperative sera (lanes 4, 6, 8, 10 and 12). This fnding suggested that the reduction of serum IGFBP-3 in postoperative serum was due to the proteolysis of IGFBP-3, and this proteolytic activity is present only in postoperative serum. To determine the susceptability of IGFBP-3 protease activity to protein inhibitors, we did the same protease assay with various protease inhibitors. Postoperative serum alone (Fig. 7, lane 2) significantly proteolysed IGFBP-3. This protease activity was completely inhibited by aprotinin, PMSF, and EDTA (lanes 3-5), but not by pepstatin, and leupeptin (lanes 6 and 7).

DISCUSSION

Gastric cancer is the leading cause of death by malignant neoplasm in Korea. Several studies demonstrated that insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) are produced by the tumor cell lines, in some of which IGFs are hypothesized to act as autocrine growth factor (16).

In the present study, we observed the alteration of serum IGF concentration. Serum IGF-I level was significantly decreased and serum IGF-II was slightly increased. The low serum IGF-I level detected in patients with gastric cancer could be due to decreased synthesis, increased catabolism of IGF-I or both. The principal regulators for IGF-I production are growth hormone (21) and nutritional status (22). Serum IGF-I levels are decreased in growth homone deficiency, protein-calorie malnutrition, fasting, severe insulin deficiency, liver disease and hypothyroidism (23). Since our patients did not show severe malnutrition or liver dysfunction, it is unlikely that a negative nitrogen balance alone can explain the decreased serum IGF-I level. Daughaday (16) observed increased serum concentration of IGF-II in several mesenchymal tumors, and most of the IGF-II was present in the state of large MW as big IGF-II. The big IGF-II fails to form 150 kDa complex and increases serum IGF-II bioactivity. This result may suppress IGF-I secretion by the liver, and explain the low serum IGF-I level. In addition, IGFs are bound to IGFBPs in equimolar ratio, decreased level of IGF-I may induce the elevation of serum IGF-II.

Serum IGFs levels and 150 kDa IGF-IGFBP complex were markedly decreased after surgery. As most serum IGFs exist as 150 kDa complex, consisting of IGFs, IGFBP-3 and ALS, reduction of this complex could result in decreased IGFs level in postoperative sera. In addition, gastric cancer patients who recieved gastrectomy did not take food for 6 days after surgery. Therefore, fasting is also an important factor in the decreased level of serum IGF-I and IGF-II after surgery.

Since most IGFs are bound in the 150 kDa complex, and serum IGF-I level was decreased in gastric cancer patients, we suggest that serum IGFBPs profile, especially IGFBP-3, may be altered. Plasma IGFBP-3 levels vary with the developmental age and the growth hormone / IGF-I, but show little diurnal or acute metabolic regulation (24). In our study, we could not find significant changes of serum IGFBPs profile in patients with gastric cancer. However, serum IGFBP-3 levels were significantly decreased 1 day after surgery and incubation of postoperative sera with control sera reduced the IGFBP-3 band. The decreased levels of serum IGFBP-3 after surgery were returning torward normal 7 days after surgery. The increase IGFBP-3 levels on the 7th day after surgery seemed to be related to refeeding. Fasting may contribute to the reduction of serum IGFBP -3 level after surgery. Clemmons et al.(25) reported only a modest decrease of IGFBP-3 in protein restricted rats.

Davies et al.(11) reported that IGFBP-3 decreases postoperatively in severely ill patients undergoing major abominal surgery. In addition, we could not observe any marked reduction of IGFBP-3 in healthy adults who fasted for 2 days. These findings suggest that the reduction of IGFBP-3 in postoperative serum mainly due to proteolysis of IGFBP-3 by surgery associated IGFBP-3 protease. This protease activity was totally inhibited by aprotinin, PMSF and EDTA but not by pepstatin and leupeptin. Recently various proteases capable of cleaving IGFBP-3 have been reported (8, 10, 26). The proteolytic activities in pregnancy serum are inhibited by aprotinin, EDTA and PMSF, a pattern consistant with cation-dependent serine protease. Therefore, these surgery induced proteases may be similar or identical. However, all IGFBP-3 proteases are not identical. Prostate specific antigen from seminal fluid (17) or urinary IGFBP-3 protease from chronic renal failure (9) has a different pattern of inhibition by protease inhibitors and produce different fragments of IGFBP-3.

Insulin-like growth factors bound in IGF-IGFBP complexes are unable to bind to their plasma membrane receptors (27). For those situations in which IGFs bound to IGFBPs are biologically inactive, dissociation of the IGF-IGFBP complexes would be necessary for IGF bioactivity to occur. Although the exact role of these proteases was not established, marked decrease of intact IGFBP-3 after surgery may make IGF-I more available for tissue uptake or for degradation. In our study, we found signficantly elevated free IGF-I levels in postoperative serum.

In conclusion, patients with gastric cancer had low serum levels of IGF-I and normal IGFBPs profile. The serum IGFBP-3 was markedly decreased after surgery and this reduction of serum IGFBP-3 was mainly due to surgery associated IGFBP-3 protease. This protease activity was totally inhibited by aprotinin, EDTA and PMSF. We speculate that surgery associated IGFBP-3 protease activity may contribute to increase the bioavailability of IGFs.

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