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European Journal of Cancer 37 (2001) 2257–2263

European Journal of Cancer

www.ejconline.com

Expression of the insulin-like growth factors (IGFs) and the IGF-binding proteins (IGFBPs) in human gastric cancer cells

H.K. Yi[a,b], P.H. Hwang[a,b], D.-H. Yang[b,c], C.-W. Kang[d], D.-Y. Lee[a,b,*]

[a]*Department of Pediatrics, Chonbuk National University, Chonju 561-712, South Korea*
[c]*General Surgery, Chonbuk National University, Chonju 561-712, South Korea*
[b]*Research Institute of Clinical Medicine, Chonbuk National University Medical School, Chonbuk National University, Chonju 561-712, South Korea*
[d]*Department of Physiology, College of Veterinary Medicine, Chonbuk National University, Chonju 561-712, South Korea*

Received 14 November 2000; received in revised form 23 April 2001; accepted 24 June 2001

Abstract

Insulin-like growth factor (IGF)-I and -II are potent mitogens and postulated to exert autocrine, and paracrine effects on growth regulation in human gastric cancer. Their mitogenic effects are tightly regulated by the IGF binding proteins (IGFBPs). In this study, we evaluated the mRNA expression of *IGF-I*, *IGF-II* and the IGFBPs in a panel of human gastric cancer cell lines, and normal and tumour tissue specimens from patients with gastric cancer by reverse transcriptase-polymerase chain reaction (RT-PCR) and competitive PCR. Conditioned media (CM) of the gastric cancer cell lines were studied for the secretion of the IGFBPs by western ligand blot (WLB) and western immunoblot (WIB). *IGF-I* and *IGF-II* were expressed in all of the gastric cancer cell lines, and the normal and tumour tissue specimens. Overexpression of the IGFs, in particular, *IGF-II*, was observed in the tumour tissues. The expression pattern of IGFBPs was heterogeneous among the gastric cancer cell lines. *IGFBP-2* was expressed in all of the gastric cancer cell lines, whereas *IGFBP-1* was not detected in any cell lines. *IGFBP-4* was expressed in the most of cell lines. *IGFBP-3*, *IGFBP-5* and *IGFBP-6* were expressed in approximately 50% of cell lines. In addition, exogenous IGF-I and IGF-II stimulated the proliferation of gastric cancer cells, suggesting the existence of a functional IGF system in gastric cancer. Taken together, our data-suggest that the IGF-IGFBP system may play an important role in the initiation, progression and metastasis of gastric cancer. Further studies are needed to understand the exact role of IGFs and IGFBPs in gastric neoplasia.

Keywords: IGF-I and IGF-II; IGFBPs; Expression; Growth; Gastric cancer

1. Introduction

Insulin-like growth factors, IGF-I and IGF-II, are potent mitogens for many cell types in the autocrine, paracrine and endocrine pathways [1]. The mitogenic effect of IGFs are specifically mediated by binding to the cell-surface IGF-I receptor (IGF-IR) and thereby, activating the receptor tyrosine kinase [2]. The biological activities of the IGFs are modulated by IGF-binding proteins (IGFBPs) which have higher affinity for IGFs than for the IGF receptors [3]. To date, six distinct high affinity IGFBPs (IGFBP-1 to -6) have been cloned and sequenced [4].

Numerous studies have demonstrated a significant role for the IGF system in controlling tumour growth [5,6]. Many tumours overexpress IGF-II, IGF-IR, and certain IGFBPs. IGF-II expression leads to an autocrine feedback loop stimulating the IGF-IR to stimulate cancer cell proliferation. The production of IGFBPs by tumour cells enhances the effects of the IGFs, thereby increasing cancer cell proliferation [7]. Conversely, IGFBPs inhibit the IGF-induced biological effects by binding to IGFs, thereby blocking IGF binding to the receptors [8]. Furthermore, IGFBPs may play more active role in cell growth. IGFBP-3, for example, has been reported to stimulate apoptosis without interacting with the IGF-IGF receptor axis in breast and prostate cancer cells [9,10].

Gastric cancer is one of the leading causes of cancer death throughout the world, although its incidence has

* Corresponding author. Tel.: +82-63-250-1469; fax: +82-63-250-1464.
E-mail address: leedy@moak.chonbuk.ac.kr (D.-Y. Lee).

0959-8049/01/$ - see front matter
PII: S0959-8049(01)00269-6

declined in many countries. The aetiology of gastric cancer is still unclear. However, the remarkable technical advances in molecular biology seen in recent years have enhanced the understanding of carcinogenesis and the progression of cancer. Normal growth and the differentiation of cells in the gastrointestinal tract is regulated by autocrine and paracrine secretion of peptide growth factors which are responsible for controlling maturation, differentiation and apoptosis [11]. In gastric neoplasia, where there is unrestricted growth, it is likely that there are abnormalities of secretion of or response to those peptides. IGFs and their receptors may be important in the regulation of epithelial cell growth, but few data are available on the expression and biological function of the IGF system in gastric cancer [12,13]. IGF-I was shown to be a potential factor for the direct regulation of proliferation of canine fundic epithelial cells in short-term cultures [14]. Durrant and colleagues [13] reported that IGF-I promotes the growth of gastric cancer cells *in vitro*.

In this study, we examined the expression of IGF-I, IGF-II and IGFBP-1 to -6 in a panel of gastric cancer cell lines derived from Koreans and Caucasians, as well as normal and tumour tissue specimens from patients with gastric cancer.

2. Materials and methods

2.1. Materials

Cell culture media (Roswell Park Memorial Institute (RPMI) 1640) and reagents were purchased from Gibco BRL (Grand Island, NY, USA). Recombinant human IGF-I and IGF-II were obtained from Bachem (Toranced, CA, USA). IGF-I was iodinated by a modification of the chloramine-T method, as previously described in Ref. [15], to specific activities of 150–300 uCi/ug. Polyclonal anti-IGFBP-3 antibody was kindly provided by Dr Ron G. Rosenfeld (Oregon Health Science University, OR, USA). Polyclonal anti-IGFBP-2 and-4 antibodies were purchased from Diagnostic System Laboratories (Webster, TX, USA).

2.2. Tissue samples

Gastric cancer specimens and adjacent normal gastric tissues were obtained from Department of Surgery, Chonbuk National University Hospital. At the time of surgery, the pathologist dissected the tumour and provided a sample of fresh non-necrotic tumour tissue. Among 10 tumour specimens, nine specimens were tubular adenocarcinomas and one was a miscellaneous carcinoma. Tissue fragments were frozen in liquid nitrogen immediately after surgery and stored at −70 °C until RNA extraction.

2.3. Cell lines and cell culture

The established human gastric carcinoma cell lines derived from Koreans were purchased from the Korea Cell Line Bank (KCLB) [16]. The designation of the various cell lines and the type of tumour are listed in Table 1. AGS, a gastric adenocarcinoma cell line derived from Caucasians, was purchased from the American Type Culture Collections (ATCC) (Manassas, VA, USA). All cell lines were maintained in RPMI supplemented with 300 ug/ml L-glutamine and 10% fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The medium was changed twice a week.

2.4. Cell proliferation assay

For the cell proliferation studies, cells were plated in 10% FBS-RPMI with 300 ug/ml L-glutamine and 25 mM HEPES buffer at a density of 3×10^4 cells/well in six-well plates. After a 12-hour incubation, the medium was changed to serum free-RPMI for 12 h, then IGF-I or IGF-II (50 and 100 ng/ml) were added at 24-h intervals

Table 1
Summary of IGF-I, -II and IGFBP-1 to -6 expressions in gastric cancer cell lines

Cell line	Histopathology	IGF-I	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
SNU-1	Adenocarcinoma	+	+	–	+	+	–	+	–
SNU-5	Adenocarcinoma	+	+	–	+	+	+	–	+
SNU-16	Adenocarcinoma	+	+	–	+	–	+	–	–
SNU-216	Adenocarcinoma	+	+	–	+	+	+	+	+
SNU-484	Adenocarcinoma	+	+	–	+	+	+	+	–
SNU-601	Carcinoma, signet ring	+	+	–	+	–	–	–	–
SNU-620	Adenocarcinoma	+	+	–	+	–	+	–	+
SNU-638	Adenocarcinoma, metastatic	+	+	–	+	–	+	+	+
SNU-668	Adenocarcinoma, signet ring	+	+	–	+	+	+	+	+
SNU-719	Adenocarcinoma	+	+	–	+	–	+	–	–
AGS	Adenocarcinoma	+	+	–	ND	–	+	ND	ND

IGF, insulin-like growth factor; IGFBP, IGF-binding protein; ND, not done.

for 3 days. The cells were trypsinised and counted using a haemocytometer on days 1, 2 and 3 after IGF treatment. Cell viability was assessed by a trypan blue exclusion assay and found to be >95%. All experiments were performed duplicate.

2.5. Preparation of conditioned media (CM) from cell culture

Cells were grown in 10% FBS-RPMI with 300 ug/ml L-glutamine and 25 mM HEPES buffer in 12-well plates. At 95% confluency, cells were washed with RPMI and the medium was changed to RPMI with additives. CM was collected after 24, 48 and 72 h and centrifuged at 1000*g* for 10 min to remove debris. The harvested CM from duplicate wells within each experiment were pooled, concentrated five times and stored at −20 °C until assay.

2.6. Western ligand blot analysis

Proteins from CM samples were size-fractionated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under non-reducing conditions and electroblotted onto nitrocellulose filters (Hybond; Amersham Pharmacia Biotech, Piscataway, NJ, USA). Filters were washed in 3% Nonidet P (NP)-40/deionized distilled (dd) H_2O for 30 min, blocked with 1% bovine serum albumin (BSA)/TBS-T (20 mM Tris–Cl, pH 7.6, 150 mM NaCl, 0.1% Tween-20) for 2 h, and incubated overnight with 2.0×10^6 counts per minute (cpm) of [^{125}I]-labelled IGF-I. The membranes were washed, dried and exposed to film (Kodak BioMax MS, Eastman Kodak Co, Rochester, NY, USA) for 5–7 days. For IGFBP-3, -2 and -4 detection, CM samples were separated on non-reducing 12% gels by SDS–PAGE. Proteins were electroblotted onto nitrocellulose, and the membranes were blocked with 3% non-fat milk/TBS-T for 1 h at room temperature, then incubated in 1:3000 dilution of primary antibodies for 2 h. Immunoreactive proteins were detected using enhanced chemiluminescence (NEN, Boston, MA, USA).

2.7. RNA extraction and RT-PCR

For total RNA preparation, cells were treated with Trizol (Life Technologies, Grand Island, NY, USA) and tissues were homogenised with a Polytron homogeniser in TRI reagent (MRC, Cincinnati, OH, USA), and total RNA was extracted according to the manufacturer's protocol. RNA samples were quantitated by spectrophotometry at 260 nm. Reverse transcription (RT) was performed in a final volume of 20 μl with 4 μg RNA and 200 U murine leukaemia virus transcriptase (Gibco BRL, Grand Island, NY, USA) at 42 °C for 60 min. Polymerase chain reaction (PCR) amplification was performed with 5 μl of RT product, 10 pmol of each specific primer, 1.25 U *Taq* polymerase (Promega) and 1 mM deoxynucleotide triphosphate (dNTP). Initial incubation at 95 °C for 3 min was followed by 35 cycles with denaturation at 95 °C for 1 min, annealing at 52 to 60 °C for 1 min, depending upon specific primers, and extension at 72 °C for 1 min. The final elongation step was extended to 10 min at 72 °C. The products were electrophoresed in 1×tris-borate-EDTA electrophoresis (TBE) buffer on 2% agarose gels containing ethidium bromide. Primer sequences were: (1) *IGF-I* sense, 5′-CAC AGG GTA TGG CTC-3′; antisense, 5′-CTT CTG GGT CTT GGG-3′; (2) *IGF-II* sense, 5′-CGA TGC TGG TGC TTC TCA-3′; antisense, 5′-GGG GTC TTG GGT GGG TAG-3′; (3) *IGFBP-1* sense, 5′-TAG TGC TCC TGC TGA CTG-3′; antisense, 5′-AGT TAT CTC CGT GCT CTC-3′; (4) *IGFBP-2* sense, 5′-CGC AAG CCC CTC AAG TCG-3′; antisense, 5′-GCC TCC TGC TGC TCA TTG-3′; (5) *IGFBP-3* sense, 5′-CTC TCC CAG GCT ACA CCA-3′; antisense, 5′-GAA GTC TGG GTG CTG TGC-3′; (6) *IGFBP-4* sense, 5′-GCG ACG AAG CCA TCC ACT-3′; antisense, 5′-TTC ATC TTG CCC CCA CTG-3′; (7) *IGFBP-5* sense, 5′-AAG AAGGAC CGC AGA AAG-3′; antisense, 5′-GGG GAC GCA TCA CTC AAC-3′; (8) *IGFBP-6* sense, 5′-GGA GTG CGG GGT CTA CA-3′; antisense, 5′-GCT TCC ATT GCC ATC TG-3′. *IGF-I* primer used in this study can measure both *IGF-Ia* and *IGF-Ib* mRNAs. *IGF-II* primer can measure all transcripts of *IGF-II* mRNA, irrespective of the types of the promoter.

3. Results

3.1. Gastric cancer cells express IGF-I and IGF-II, and certain IGFBPs

In this study, we examined the expression pattern of the IGFs and IGFBPs by the use of RT-PCR in the gastric cancer cell lines. As can be seen in Fig. 1, both

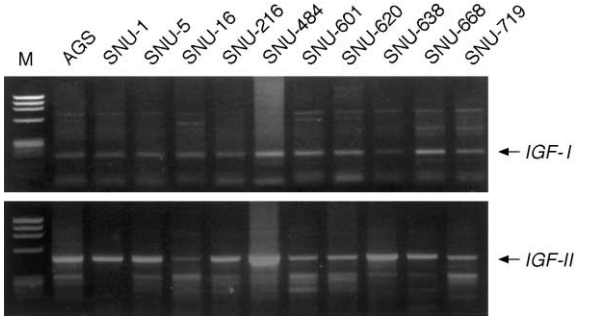

Fig. 1. Expression of insulin-like growth factor (*IGF*)-*I* and *IGF-II* mRNA in human gastric cancer cells by reverse transcriptase-polymerase chain reaction (RT-PCR). A representative blot from three separate experiments is shown. Lane 1, molecular marker; lane 2, AGS control; lanes 3–12, 10 Korean gastric cancer cell lines.

IGF-I and *IGF-II* were expressed in all of the Korean gastric cancer cell lines, as well as in the AGS cell line, albeit at different levels among the cell lines tested. The expression pattern of the IGFBP species was heterogeneous among the cell lines. *IGFBP-1* mRNA was not detected in any cell lines, whereas *IGFBP-2* was expressed in all of the Korean gastric cancer cell lines (Fig. 2). *IGFBP-3* expression was found in five out of 11 cell lines (SNU-1, -5, -216, -484 and -668), and *IGFBP-4* mRNA was detected in most of 11 cell lines (except SNU-1 and -601). *IGFBP-5* and *IGFBP-6* were expressed in approximately 50% of gastric cancer cell lines. Table 1 summarises the IGFs and IGFBPs mRNA expression in the gastric cancer cell lines we have examined.

3.2. Gastric cancer cells produce various IGFBPs

Since IGFBP-expressing cell lines have been shown to secrete IGFBPs into the CM, we performed a Western ligand blot (WLB) analysis of CM after 24, 48 and 72 h serum-free condition. As shown in Fig. 3, two species of IGFBP was readily detectable and further characterisation with specific antibodies for IGFBPs 1–4 revealed that the 42-kDa species represents IGFBP-3, whereas the 30-kDa species represents IGFBP-2 (data not shown). Even if IGFBP-4 was confirmed with polyclonal anti-IGFBP-4 antibody in the SNU-638 cells, the IGFBP-4 band was hardly detected on WLB. In agreement with the mRNA data, IGFBP-1 was not detected in any samples tested by use of specific polyclonal antibodies. The levels of the IGFBP-3 and -2 proteins in the CM were increased in a time-dependent manner up to 72 h.

3.3. IGF-I *and* IGF-II *stimulate gastric cancer cell growth*

To test whether the IGFs are potent mitogens in gastric cancer cells, a cell proliferation assay was performed with treatment of various concentrations of IGFs in SNU-638 and AGS cells (Fig. 4). Treatment with IGF-I (50 or 100 ng/ml) for 72 h resulted in a 0.5–1.1-fold increase in SNU-638 cell proliferation, compared with controls ($P<0.05$), and showed a dose-dependent growth response (Fig. 4a). IGF-II-treated cell growth was similar to that of the IGF-I-treated cell, but IGF-II was a more potent stimulator of SNU-638 cell growth than IGF-I. Similar growth effect of the IGFs were observed in the AGS cells (Fig. 4b).

3.4. Gastric cancer tissues express IGF-I *and* IGF-II

Since increased expression of *IGF-I* and *IGF-II* mRNA have been detected in numerous human tumour tissues, we tested *IGF-I* and *IGF-II* expressions in both normal and cancer tissues from 10 individual patients with gastric cancer (P1–P10). As seen in Fig. 5, *IGF-I* and *IGF-II* were expressed in all normal tissues, as well as in gastric cancer tissues. In addition, we found that *IGF-I* expression was increased in 2 cases of gastric tubular adenocarcinoma with moderate differentiation (P1, P2), and *IGF-II* expression was increased in 9 cases of gastric tubular adenocarcinoma (except P4) compared with normal gastric tissues.

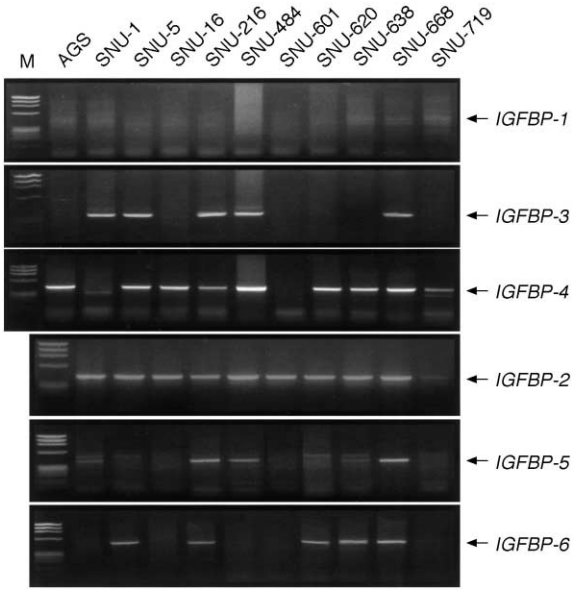

Fig. 2. Expression of IGF-binding protein (*IGFBP*)-*1* to *IGFBP-6* mRNA in human gastric cancer cells by RT-PCR. A representative blot from three separate experiments is shown as described in Fig. 1.

4. Discussion

It is apparent that autocrine, paracrine and endocrine-derived growth factors play an important role in tumorigenesis, tumour growth and progression *in vivo* [17]. To date, it has been reported that five principal families of peptide growth factors are critically involved in gastric carcinogenesis; the transforming growth

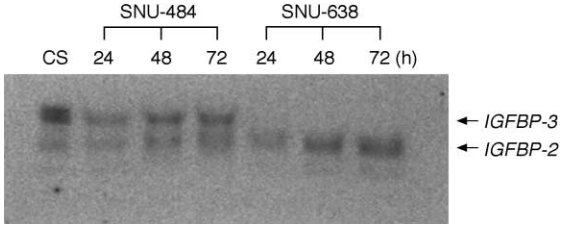

Fig. 3. Production of IGFBP proteins in conditioned media (CM) from gastric cancer cells. Representative western ligand blot derived from three independent experiments is shown. CM collected after 24, 48 and 72 h in serum-free conditions was blotted to a nitrocellulose membrane and was probed with a [^{125}]I-labelled IGF-I as indicated in Materials and methods. Lane 1, adult human serum (CS); lanes 2–4, SNU-484; lanes 5–7, SNU-638.

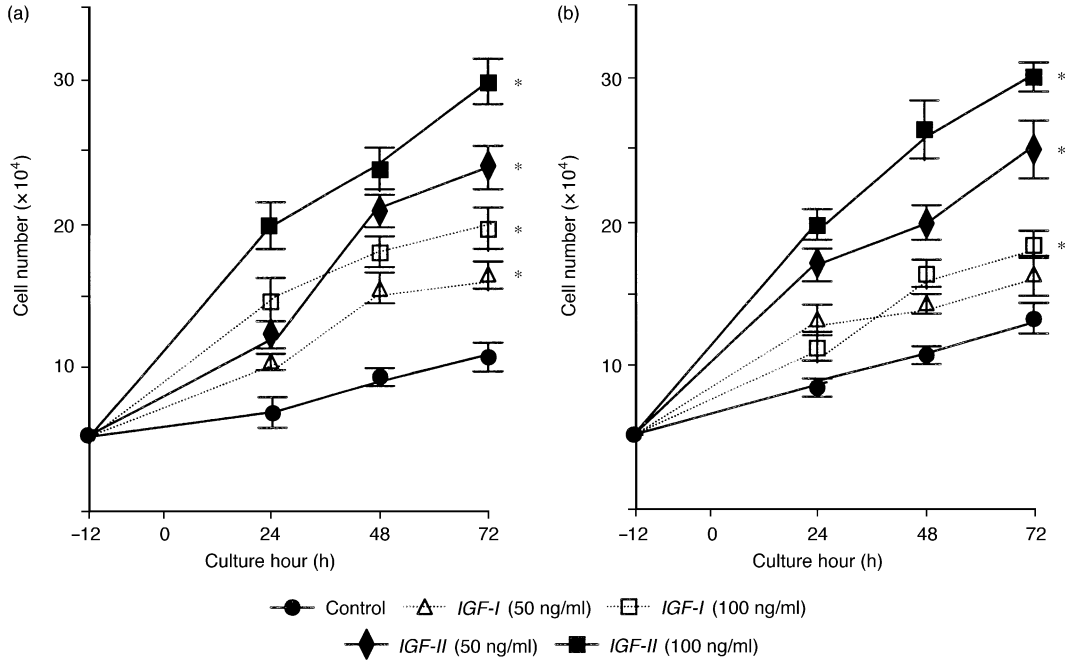

Fig. 4. Effects of IGF-I and IGF-II on proliferation of (a) SNU-638 and (b) AGS cells grown under serum-free conditions for 3 days. The figure shows one representative experiment of two. Cells were treated with 50 or 100 ng/ml of IGF-I and IGF-II. Results represent the mean±standard error of the mean (S.E.M.) of cell number in triplicate wells. Means were compared by ANOVA using Scheffe's method. *P values of <0.05 (compared with control) were considered significant.

factor TGF-β family [18], epidermal growth factor family [19], IGFs [12], fibroblast growth factor family [20], and hepatocyte growth factor [21]. Numerous studies have demonstrated that IGFs and IGFBPs are produced by tumour cell lines and tumours, acting as an autocrine growth factor [5–7].

In this study, we have investigated the expression of IGFs and IGFBPs in gastric cancer cell lines in detail to predict the potential role of the IGF-IGFBP system in the development and growth of gastric cancer. We also studied IGF-I and -II expression in human gastric normal and cancer tissues. IGF-I and -II were expressed in all Korean gastric cancer cell lines as well as in AGS cells. In addition, IGF-I and -II stimulated the proliferation of SNU-638 and AGS cells. Our data suggest that IGF-I and -II function as autocrine growth factors in gastric cancer. Moreover, recent studies suggest that IGF-I and -II are involved in development of normal tissues, including stomach, as well as the neoplastic transformation and proliferation of cancer cells [22]. Overexpression of IGF-II appears to be strongly associated with the neoplastic conversion of epithelial cells in some cell types and cancers [23,24]. In this study, we found that IGF-II was overexpressed in 90% of the gastric cancer specimens examined. Takeshi and colleagues [25] also reported that IGF-II is overexpressed in tissues from patients with gastric cancer compared to normal gastric tissues. These data suggest that IGF-II may have an important role in to play gastric cancer development or growth.

Since bioactivity and bioavailabilty of IGFs are modulated by the IGFBPs and the expression of the IGFBPs is tightly regulated by various peptide growth factors, characterisation of the IGFBP expression will be important to understand the role of IGFs in human gastric cancer proliferation. We have shown that the

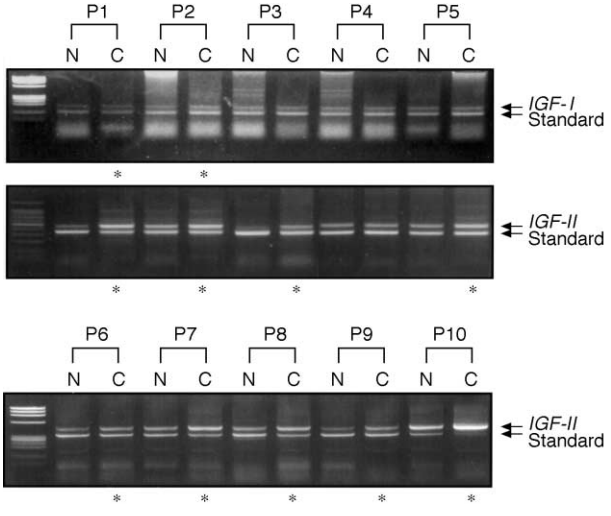

Fig. 5. Expression of *IGF-I* and *IGF-II* mRNA in human gastric cancer tissues. Total RNA from normal (N) and cancer (C) tissues from 10 individual gastric cancer patients (P1 to P10) were isolated and subjected to competitive-PCR. A representative blot from three separate experiments is shown. * Means more than 10% increase in *IGF-I* or *IGF-II* expressions compared with normal tissue.

expression of IGFBPs in gastric cancer cell lines is heterogeneous, although no single cell line apparently expresses all IGFBPs. The pattern of expression in several IGFBPs did not completely correlate with the IGFBP species detected by WLB. For example, SNU-484 cells expressed IGFBP-2, -3, -4, and -5 mRNAs. However, only two IGFBP species, IGFBP-2 and IGFBP-3, were detected in the CM. IGFBP-4 and -5 are not evident on the WLB. This might be due to either the IGFBP-4 and -5 messages not being translated or limitations in the detection level by use of WLB. In general, IGFBP-2 was expressed in all cell lines, and IGFBP-3, -5 and -6 were expressed in approximately 50% of cell lines. This pattern of expression may not exclusive to gastric cancer cells and is similar to colon cancer cells. Singh and colleagues [26] reported that most colon cancers express IGFBP-2 and IGFBP-4; less than 50% also express IGFBP-3, IGFBP-5, and IGFBP-6; and none expresses IGFBP-1. However, these patterns of expression could have biological significance and determine the cellular response to autocrine- or paracrine-derived IGFs.

The ubiquitous expression of IGFBP-2 in the panel of cell lines studied here suggests that IGFBP-2 is intimately associated with the malignant phenotype, and confers some growth advantage on the tumour cells. Several studies have suggested that IGFBP-2 expression may be associated with malignancy [27,28], but the exact role of IGFBP-2 in tumour development including gastric cancer is not known. Based on the finding that the serum level of IGFBP-2 is increased in various types of tumours, Zumkeller and colleagues [28] concluded that the serum IGFBP-2 measurement could be a useful marker for the diagnosis of various malignancies. Both stimulatory and inhibitory effects of IGFBP-2 on IGF activity have been reported, depending on the model examined. IGFBP-2 may increase the proliferative effects of the IGFs by binding to the cell membrane or to the extracellular matrix, thus facilitating IGF binding to its receptor [29]. Proteolysis is another possible mechanism for regulating IGFBP-2/IGF interactions [30]. By decreasing the IGF affinity, IGFBP-2 proteolysis may increase IGF bioavailability and enhance its proliferative effects on gastric cancer cells. On the other hand, IGFBP-2 can inhibit the mitogenic effects of IGFs and this inhibitory effect has been attributed primarily to competitive scavenging of IGF peptides away from the IGF-I receptors [31]. Since gastric cancer cell lines express both mitogens and the modulatory IGFBPs, they may play an important role in the growth and differentiation of gastric cancer cells in response to endogeneous IGFs. We are expanding these studies to examine gastric cancer tissue specimens to analyse the statistical relationship between the mRNA expression of *IGF-I*, *IGF-II* and *IGFBP-1* to *IGFBP-6* genes and various clinical pathological parameters.

In conclusion, we have performed an extensive characterisation of IGF-I and IGF-II, and the IGFBPs in gastric cancer cell lines. IGF-I and -II were expressed in all of the gastric cancer cells and IGFBP expression was heterogeneous depending upon cell line, suggesting that the IGF-IGFBP system may play an important role in gastric cancer cell growth. Further studies are needed to understand the role of the IGFs and IGFBPs in the initiation, progression and metastasis of gastric cancer.

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