

Instability of IGF-IGFBP complex as a cause of the different performance of serum and EDTA-plasma after storage: EDTA-plasma is preferable for evaluating bioactive IGF especially in the mouse

Takeshi Setoyama^a, Shin'ichi Miyamoto^a, Mitsuhiro Nikaido^a, Manabu Muto^b, Tsutomu Chiba^a

^a Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, ^b Department of Therapeutic Oncology, Kyoto University Graduate School of Medicine, 54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Address correspondence to:

Shin'ichi Miyamoto, M.D., Ph.D.

Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin-kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Tel: +81-75-751-4319, Fax: +81-75-751-4303

Email: shmiyamo@kuhp.kyoto-u.ac.jp

ABSTRACT

Objective: The insulin-like growth factor (IGF) signaling pathway is recognized as a potential target for treating several cancers, and strategies targeting the IGF type 1 receptor (IGF-1R) have been evaluated in many clinical trials. These suggested that the pretreatment level of circulating free IGF gives an estimate of IGF bioactivity and might be a predictive biomarker of the response to anti-IGF-1R antibodies. However, there is no defined protocol for measuring free and bioactive IGF concentrations, partly because the measurement procedures, including sample collection and handling, have not been standardized. We investigated the effects of sample collection methods and storage conditions on bioactive IGF measurement using a modified kinase receptor activation (KIRA) assay in human and mouse samples.

Design: Blood samples were obtained from healthy men and women, and from healthy male and female wild-type BALB/c mice. Serum and ethylenediaminetetraacetic acid (EDTA)-plasma samples were collected and used immediately or stored in small quantities at 4 °C or –80 °C for 3, 7, or 14 days. A bioassay directed against the phosphorylated IGF-1R using western blot analysis was developed as a modification of the KIRA assay, in which the level of phosphorylation of IGF-1R represented the IGF bioactivity in blood samples.

Results: The levels of bioactive IGFs in mouse serum stored at 4 °C increased markedly in a time-dependent manner; the increase was slightly reduced in samples stored at –80 °C. Analysis of mouse EDTA-plasma stored at 4 °C showed a similar pattern, but the time-dependent increase was less than in the serum samples. By contrast, the levels of bioactive IGFs in EDTA-plasma stored at –80 °C were stable over 14 days. The levels of human bioactive IGFs in both serum and EDTA-plasma stored at 4 °C increased slightly with time, but the increases were much smaller than in mouse samples. The levels of human bioactive IGF in both serum and EDTA-plasma stored at –80 °C were stable over 14 days.

Conclusions: The use of EDTA-plasma avoids the problems with long-term storage. Therefore, EDTA-plasma should be used when measuring circulating IGF bioactivity, especially in mouse samples. All samples should be stored at –80 °C when long-term storage is unavoidable. Because of the large difference in the stability of the IGF–IGF-binding protein complex between the human and mouse *in vitro*, all samples should be handled carefully to ensure the accurate evaluation of IGF bioactivity, especially in mouse samples.

Keywords:

Insulin-like growth factor (IGF)

IGF-binding protein (IGFBP)

Bioactive IGF

Free IGF

Kinase receptor activation (KIRA) assay

IGF–IGFBP complex

Highlights:

Detected bioactive IGFs increased in mouse serum samples during storage.

The mouse IGF–IGFBP complex is more fragile than the human one *in vitro*.

The use of fresh EDTA-treated plasma is preferable for evaluating IGF bioactivity.

All blood samples should be stored at –80 °C when long-term storage is unavoidable.

INTRODUCTION

The insulin-like growth factor (IGF) system comprises two ligands, IGF-I and IGF-II. There are three different receptors, IGF type 1 receptor (IGF-1R), IGF type 2 receptor, and the insulin receptor, and six IGF-binding proteins (IGFBP-1 through -6). IGF-1R is the main receptor common to the two ligands involved in activation of the signaling pathway. The physiological activities of IGFs are modulated by IGFBPs, which can inhibit the signaling pathway by capturing the circulating IGFs, thus blocking their receptor binding [1–3]. The receptors are only activated by free IGFs (~1% of total IGFs) released from the IGF–IGFBP complex mainly by proteolysis of IGFBP [1–3].

The IGF system is involved in the progression of human cancers and is a potential target for therapeutic intervention [2,3]. Free IGF in its bioactive form is recognized as being critical for activation of the IGF system [4–6]. Some studies using data from clinical trials targeting IGF-1R have suggested that pretreatment levels of circulating free IGFs have potential as predictive biomarkers of the response to anti-IGF-1R antibodies [7–9]. In these clinical studies, commercially available immunoassays were used to evaluate the level of circulating free IGF [5–9]. However, these assays can be influenced by IGFBPs, thereby causing considerable differences in the results between assays, procedures, and measurement conditions [10–12]. Efforts have been made to standardize the measurement of total IGF concentration, whereas standard protocols to measure free or bioactive IGF concentrations have not yet been established [13,14]. There are no commercially available assays for evaluating the levels of free or bioactive IGFs in mouse blood, and to our knowledge only a few studies have evaluated circulating IGF bioactivity in the mouse [15]. Therefore, for clinical and experimental studies, there is a critical need to standardize the procedures used to

measure the levels of free or bioactive IGFs, including the sample collection methods and storage conditions in both human and mouse studies.

Here, we investigated the effects of sample collection methods and storage conditions on the evaluation of IGF bioactivity using the modified kinase receptor activation (KIRA) assay and examined whether there were any differences in the stability of the IGF–IGFBP complex *in vitro* between human and mouse samples.

MATERIALS AND METHODS

Sample collection and storage

To investigate the effects of the sample collection method and storage condition, blood samples were obtained from 11 healthy human subjects (six male and five female), mean age and standard deviation (SD) 35.7 ± 9.6 years, and from 21 healthy male and female wild-type BALB/c mice (age 11.3 ± 3.6 weeks: six 8-week-old male, five 15-week-old male, five 8-week-old female, and five 15-week-old female mice). To elucidate the mechanisms responsible for the increase in IGF bioactivity in stored samples, blood samples were obtained from 10 8-week-old healthy male and female wild-type BALB/c mice (four male and six female mice). All blood samples were obtained through venipuncture and were centrifuged as soon as possible at $\sim 1200 g$ for 15 min. Serum and ethylenediaminetetraacetic acid (EDTA)-plasma samples (EDTA final concentration 0.1%) were collected after centrifugation and were used immediately or stored in small aliquots at 4 °C or –80 °C for 3, 7,

or 14 days. Samples stored at -80°C went through only one freeze–thaw cycle before measurement.

The studies were conducted in accordance with the Helsinki Declaration with amendments and were approved by the animal protection committee of our institution. The animals were housed in specific pathogen-free conditions in the animal facility of Kyoto University. All mice (from Japan SLC, Hamamatsu, Japan) were housed in groups of no more than six per cage under controlled conditions (artificial light, $24 \pm 2^{\circ}\text{C}$; humidity $50 \pm 10\%$), 14-h light and 10-h dark cycle, and received standard laboratory chow throughout the study.

Procedure for the modified KIRA assay

A cell-based assay to measure bioactive IGFs by measuring the phosphorylation levels of IGF-1R was developed as a modification of the KIRA assay reported by Chen *et al.* [16]. Subconfluent embryonic mouse hypothalamic (N7) cells overexpressing the human IGF-1R (N7-IGF-1R cells) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen Life Technologies) in a 6 cm dish and then transferred to 2 mL of FBS-free DMEM at least 5 h before any stimulation by blood samples. After the FBS-free DMEM had been discarded, the N7-IGF-1R cells were stimulated by 1 mL of a blood sample diluted in Krebs–Ringer bicarbonate buffer (KRB buffer) (the protein concentration was adjusted to 1 mg/mL) and incubated for 15 min at 37°C . To inhibit the endogenous IGFBP protease activity, an EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) was added according to the manufacturer's protocol. After washing twice with ice-cold phosphate buffered saline, cells were lysed as described [17].

Each sample (10 µg of cell lysate) was fractionated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and then transferred to a polyvinylidene difluoride membrane (Pall Corp., Pensacola, FL, USA). Nonspecific binding was blocked for 1 h with 5% nonfat milk powder and 1% bovine serum albumin (BSA) in Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 at room temperature. The membrane was incubated overnight with specific rabbit polyclonal anti-phosphorylated IGF-1R antibodies (Invitrogen Life Sciences) and with anti-IGF-1R antibodies (C-20, Santa Cruz Biotechnology, Dallas, TX, USA) for measuring the total IGF-1R protein level, respectively, at 4 °C. The membrane was then incubated for 1 h with peroxidase-labeled donkey anti-rabbit antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The bands were visualized with an enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL, USA), and the image was recorded using a chemiluminescence image reader (ChemiDoc XRS plus, Bio-Rad Laboratories, Hercules, CA, USA). The integral value of the signal strengths of each visualized and recorded band was calculated as band intensity using the image analysis software Image Lab (Bio-Rad Laboratories). The band intensity obtained for the phosphorylated IGF-1R protein stimulated by each sample was normalized against the corresponding band intensity obtained for total IGF-1R protein. Furthermore, using the value calculated by subtracting the normalized phosphorylated IGF-1R protein band intensity of the negative control, stimulated by KRB buffer without any ligands, from each sample normalized against the phosphorylated IGF-1R protein band intensity could eliminate the artifacts and background intensity of western blot analyses. IGF bioactivity for 10 ng/ml of recombinant IGF-I measured in our assay was set to 1.0 for human and mouse samples, and the result obtained from each measurement is expressed as the relative phosphorylation level of IGF-1R.

Assay validation

Assay reproducibility was estimated by repeated measurements of recombinant mouse and human IGF-I. The effect of a sample dilution on the assay was also tested. Serum and EDTA-plasma obtained from a healthy 8-week-old male wild-type BALB/c mouse and a healthy 38-year-old man were diluted in KRB buffer to 0.1, 0.5, 1, and 2 mg/ml protein concentrations.

Effects of protease inhibitors during storage

To elucidate the effects of IGFBP proteolysis on the change in IGF bioactivity in stored samples, mouse sera were stored for 7 days at 4 °C in the absence or presence of two kinds of protease inhibitors, and IGF bioactivity was measured. An EDTA-free protease inhibitor cocktail (Roche) and Ilomastat, a matrix metalloproteinase (MMP) inhibitor, (GM6001; Merck Millipore, Darmstadt, Germany) were added to the sera according to the manufacturer's protocols as soon as the samples were collected.

Statistical analyses

Because the serum and EDTA-plasma bioactive IGF levels exhibited asymmetrical distributions, nonparametric tests were used. The mean levels of bioactive IGFs were compared between conditions using the Mann-Whitney *U* test. Statistical calculations were done with the StatView package (Abacus Concepts, Berkeley, CA, USA). Data are given as the mean $\pm$ standard deviation (SD). A *p*-value < 0.05 was considered significant.

RESULTS

Assay characteristics

A standard curve is shown in Fig. 1A. This was estimated by repetitive measurements of a mouse and human recombinant IGF-I assayed three times on three different days and three different western blots. Moreover, the effects of diluting mouse and human blood samples on the assay were also tested (Fig. 1B). The detected bioactivity depended on the amount of protein used for stimulation. From this we decided that N7-IGF-1R cells would be stimulated by 1 mg protein aliquots of each sample for further experiments.

Effects of storage conditions

To clarify the effect of storage conditions on the evaluation of IGF bioactivity, a modified KIRA assay was applied to detect bioactive IGFs in fresh samples and in samples that had been stored for 3, 7, or 14 days at 4 °C or –80 °C. Representative results of the modified KIRA assay of samples from an 8-week-old male mouse and a 38-year-old man are shown in Figs 2 and 3, respectively. The level of mouse serum bioactive IGFs increased markedly in a time-dependent manner in samples stored at 4 °C (Fig. 4A, black column). A similar pattern was seen in samples stored at –80 °C, although the increase was less (Fig. 4A, gray column). Analysis of mouse EDTA-plasma samples stored at 4 °C showed a similar pattern, but the time-dependent increase in the level of bioactive IGFs was much smaller than that observed for the serum

samples (Fig. 4B, black column). By contrast, the level of mouse bioactive IGFs in EDTA-plasma samples stored at -80°C was stable over the 14 days (Fig. 4B, gray column). Compared with the fresh sample, the increased rate of IGF bioactivity after 14 days of storage in serum was 308% at 4°C ($p < 0.0001$; Fig. 4A, black column) and 100% at -80°C ($p = 0.0022$; Fig. 4A, gray column); after storage in EDTA-plasma, it was 74% at 4°C ($p = 0.017$; Fig. 4B, black column) and 30% at -80°C (not significant; Fig. 4B, gray column).

In human samples stored at 4°C , the detected levels of bioactive IGF in both serum and EDTA-plasma increased slightly with time, but much less than in mouse samples (Fig. 5A, B, black column). By contrast, the detected levels of bioactive IGF in samples stored at -80°C were stable over the 14 days (Fig. 5A, B, gray column). In human samples, the storage temperature did not strongly affect the stability of the IGF-IGFBP complex during the sample collection and storage periods. Compared with the fresh sample, the increased rate after 14 days storage in serum was 76% at 4°C (not significant; Fig. 5A, black column) and 42% at -80°C (not significant; Fig. 5A, gray column); after storage in EDTA-plasma, it was 96% at 4°C ($p = 0.045$; Fig. 5B, black column) and -21% at -80°C (not significant; Fig. 5B, gray column).

Effect of protease inhibitors on the stability of the IGF-IGFBP complex during storage

To elucidate the mechanism responsible for increasing IGF bioactivity in stored samples, the modified KIRA assay was applied to detect bioactive IGFs in the absence or presence of two kinds of protease inhibitors in mouse serum samples stored for 7 days at 4°C . The assay showed that the protease inhibitors, including the MMP inhibitor, did not inhibit the increase in mouse IGF bioactivity during storage (Fig. 6).

DISCUSSION

Protocols for sample collection and handling have been developed for standardizing the measurement of total serum IGF-I concentrations [14]. By contrast, no standard protocols for measuring free and bioactive IGF concentrations have been established. There are three possible reasons for the lack of standardization. First, the half-life of free IGF is about 15 min, and free IGF is unstable under physiological conditions [18]. Second, the optimum preanalytical procedures to detect free IGF have not been investigated fully. Third, commercially available immunoassays to measure the free IGF concentration might need further validation because most of them ignore the modifying effects of IGFBPs on the interaction between IGFs and IGF-1R [11,12,16].

Under these circumstances, other assays have been developed to improve the estimation of IGF bioactivity *in vivo* [13,16]. Chen *et al.* developed a KIRA assay to evaluate IGF bioactivity in individual blood samples under physiological conditions *in vitro* [16]. In the present study, we simplified this. The conventional KIRA was reported to use both an anti-IGF-1R antibody and a monoclonal anti-phosphotyrosine antibody to detect phosphorylated receptors [16]. On the other hand, in our assay, with an anti-phosphorylated receptor antibody, phosphorylated IGF-1R could be detected directly so the detection might be more accurate and simpler. Although the methodology might not represent *in vivo* conditions, the conventional KIRA and our modified method should reflect the IGF activity more correctly in a sample than other methods [13]. In our assay, IGF-II is thought to contribute to the present assays, especially in human samples. The IGF bioactivity detected might represent whole bioactivity combined of both IGF-I and IGF-II, and each ligand's activity was not distinguished by the

present assay. And, the calculated coefficients of variation of the standards in 1.0 to 20 ng/ml of the concentration averaged 17%. On the other hand, reproducibility was lacking in the concentration of 0.1 and 0.5 ng/ml. These results are inferior to the conventional KIRA (16). However, we could evaluate the IGF bioactivity of the sample as a relative value compared each other or to the control, “fresh sample without storage”. Thus, the present method is thought to be scientifically valid to draw our conclusion. In order to measure the bioactive IGFs more correctly, cell-based ELISA to evaluate the total and phosphorylated IGF-1R simultaneously is currently under investigation.

Using this modified KIRA assay, we clarified that the use of EDTA-plasma avoids the problems associated with long-term storage and that EDTA-plasma should be used when evaluating circulating IGF bioactivity, especially in mouse samples. In fresh samples, there was no significant difference between serum and EDTA-plasma. However, in the mouse, the levels of IGF bioactivity increased more with the duration of storage in the serum compared with EDTA-plasma. The IGF–IGFBP complex was more stable in human samples *in vitro* than in mouse samples. The IGF bioactivity in mouse circulation has been hardly studied so far. To our knowledge, this is the first report about the large difference in the stability of the IGF–IGFBP complex between the human and mouse. Although the mechanism responsible for this difference between the human and mouse is unclear, our data suggest that all samples should be handled carefully to ensure the accurate evaluation of IGF bioactivity, especially in mouse samples.

The time-dependent increase in bioactivity we found might reflect increasing levels of free IGFs released from the IGF–IGFBP complex. A previous study reported that free IGF level increased with storage at room temperature and suggested that free IGF dissociates from its IGFBPs during storage [19]. Another report suggested that the slow degradation of IGFBPs in

EDTA-plasma might lead to discrepancies in the measured values of IGFBPs between serum and EDTA-plasma, even in specimens collected from the same individuals [20]. In the present study, protease inhibitors, including an MMP inhibitor, did not inhibit the increase in IGF bioactivity, which suggests that the dissociation of free IGF from the IGF–IGFBP complex rather than proteolysis of IGFBP might have been responsible. However, further studies are needed to elucidate the precise mechanisms responsible for the dissociation of free IGF from the IGF–IGFBP complex.

Interestingly, some studies using data from clinical trials targeting IGF-1R have suggested that the pretreatment level of circulating free IGFs may be a predictive biomarker of the response to anti-IGF-1R antibodies [7–9]. We have previously developed IGF-neutralizing antibodies and have reported on the therapeutic efficacy of these antibodies in treating liver metastases of colorectal cancers [21], bone metastases of prostate cancer, breast cancer [22,23], and multiple myeloma [24]. In theory, the circulating levels of bioactive IGFs might be more promising predictive biomarkers of the response to IGF–ligand-targeting strategies compared with IGF-1R-targeting strategies.

In conclusion, the evaluation of circulating IGF bioactivity appears to be strongly influenced by preanalytical procedures. Our data suggest that fresh EDTA-plasma is preferable for evaluating the level of bioactive IGFs accurately—especially for the mouse—and that all samples should be stored at –80 °C when long-term storage is unavoidable.

DISCLOSURE STATEMENT

All authors report no conflict of interest.

ACKNOWLEDGMENTS

We thank Dr. Axel Ullrich for generously providing the N7-IGF-1R cells and Dr. Jan Frystyk for useful advice about the IGF bioassay. We also thank Mihoko Tsurumaki and Yukie Nakai for technical assistance.

REFERENCES

- [1] S.M. Firth, R.C. Baxter, Cellular actions of the insulin-like growth factor binding proteins, *Endocr. Rev.* 23 (2002) 824–854.
- [2] M. Pollak, E.S. Schernhammer, S.E. Hankinson, Insulin-like growth factors and neoplasia, *Nat. Rev. Cancer* 4 (2004) 505–518.
- [3] M. Pollak, The insulin and insulin-like growth factor receptor family in neoplasia: an update, *Nat. Rev. Cancer* 12 (2012) 159–169.
- [4] J. Frystyk, Utility of free IGF-1 measurement, *Pituitary* 10 (2007) 181–187.
- [5] M.J. Gunter, D.R. Hoover, H. Yu, et al., Insulin, insulin-like growth factor-I, and risk of breast cancer in postmenopausal women, *J. Natl. Cancer Inst.* 101 (2009) 48–60.
- [6] M.J. Gunter, D.R. Hoover, H. Yu, et al., Insulin, insulin-like growth factor-I, endogenous estradiol and risk of colorectal cancer in postmenopausal women, *Cancer Res.* 68 (2008) 329–337.
- [7] H. Juergens, N.C. Daw, B. Geoerger, et al., Preliminary efficacy of the anti-insulin-like growth factor type 1 receptor antibody figitumumab in patients with refractory Ewing sarcoma, *J. Clin. Oncol.* 29 (2011) 4534–4540.
- [8] A. Gualberto, M.L. Hixon, D.D. Karp, et al., Pre-treatment levels of circulating free IGF-I identify NSCLC patients who derive clinical benefit from figitumumab. *Br. J. Cancer* 104 (2011) 68–74.
- [9] A. Weickhardt, R. Doebele, A. Oton, et al., A phase I/II study of erlotinib in combination with the anti-insulin-like growth factor-1 receptor monoclonal antibody IMC-A12 (Cixutumumab) in patients with advanced non-small cell lung cancer, *J. Thorac. Oncol.* 7 (2012) 419–426.

- [10] J. Frystyk, P. Ivarsen, R.K. Stoving, et al., Determination of free insulin-like growth factor-I in human serum: comparison of ultrafiltration and direct immunoradiometric assay, *Growth Horm. IGF Res.* 11 (2001) 117–127.
- [11] P. Bang, M. Ahlsen, U. Berg, et al., Free insulin-like growth factor I: are we hunting a ghost? *Horm. Res.* 55 (2001) 84–93.
- [12] J.A. Janssen, Insulin-like growth factor I: pros and cons of a bioassay, *Horm. Res. Paediatr.* 76 (2011) 106–110.
- [13] R. Hjortebjerg, J. Frystyk, Determination of IGFs and their binding proteins, *Best Pract. Res. Clin. Endocrinol. Metab.* 27 (2013) 771–781.
- [14] D.R. Clemmons, Consensus statement on the standardization and evaluation of growth hormone and insulin-like growth factor assays, *Clin. Chem.* 57 (2011) 555–559.
- [15] S. Elis, Y. Wu, H-W. Courtland, et al., Unbound (bioavailable) IGF1 enhances somatic growth, *Dis. Model. Mech.* 4 (2011) 649–658.
- [16] J.W. Chen, T. Ledet, H. Orskov, et al., A highly sensitive and specific assay for determination of IGF-1 bioactivity in human serum. *Am. J. Physiol. Endocrinol. Metab.* 284 (2003) 1149–1155.
- [17] S. Miyamoto, K. Yano, S. Sugimoto, et al., Matrix metalloprotease-7 facilitates insulin-like growth factor bioavailability through its proteinase activity on insulin-like growth factor binding protein 3. *Cancer Res.* 64 (2004) 665–671.
- [18] H.P. Guler, J Zapf, C. Schmid, et al., Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates, *Acta Endocrinol. (Copenh)* 121 (1989) 753–758.
- [19] T.G. Harris, H.D. Strickler, H. Yu, et al., Specimen processing time and measurement of total insulin-like growth factor-I (IGF-I), free IGF-I, and IGF binding protein-3 (IGFBP-3), *Growth Horm. IGF Res.* 16 (2006) 86–92.

- [20] H. Yu, J. Mistry, M.J. Nicar, et al., Insulin-like growth factors (IGF-I, free IGF-I, and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation, *J. Clin. Lab. Anal.* 13 (1999) 166–172.
- [21] S. Miyamoto, M. Nakamura, K. Shitara, et al., Blockade of paracrine supply of insulin-like growth factors using neutralizing antibodies suppresses the liver metastasis of human colorectal cancers, *Clin. Cancer Res.* 11 (2005) 3494–3502.
- [22] M. Goya, S. Miyamoto, K Nagai, et al., Growth inhibition of human prostate cancer cells in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice by a ligand-specific antibody to human insulin-like growth factors, *Cancer Res.* 64 (2004) 6252–6258.
- [23] T. Sangai, H. Fujimoto, S Miyamoto, et al., Roles of osteoclasts and bone-derived IGFs in the survival and growth of human breast cancer cells in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice, *Clin. Exp. Metastasis* 25 (2008) 401–410.
- [24] K. Araki, T. Sangai, S. Miyamoto, et al., Inhibition of bone-derived insulin-like growth factors by a ligand-specific antibody suppresses the growth of human multiple myeloma in the human adult bone explanted in NOD/SCID mouse, *Int. J. Cancer* 118 (2006) 2602–2608.

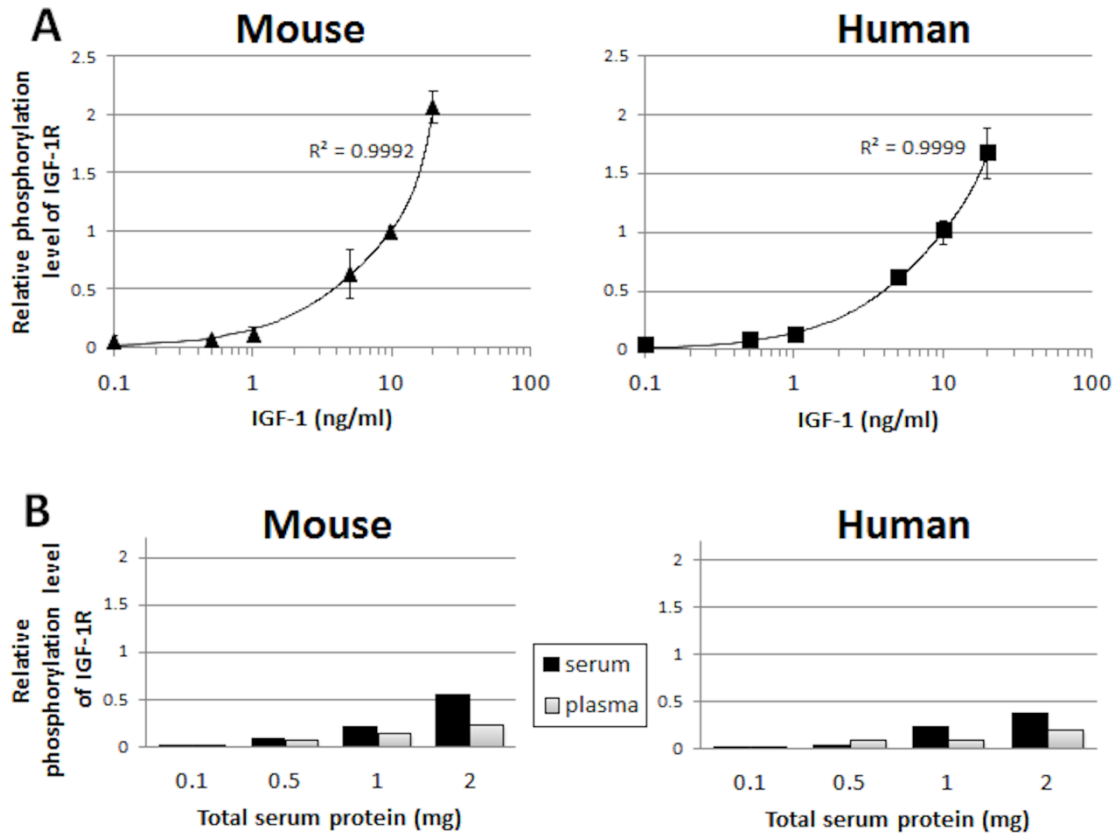
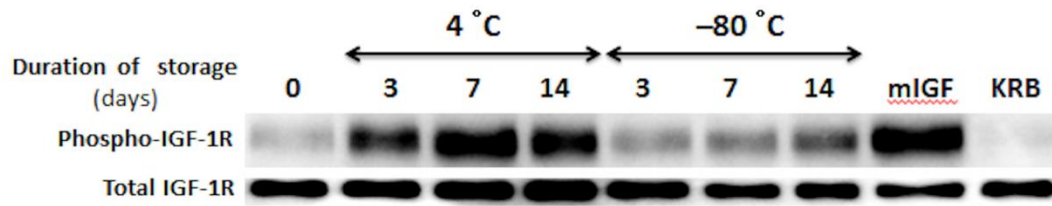


Fig. 1. (A) Dose-response curves for recombinant mouse ($\blacktriangle$) and human ($\blacksquare$) IGF-I. Recombinant mouse and human IGF-I phosphorylated IGF-1R in a dose-dependent manner. Data are means $\pm$ SDs. (B) Effects of different dilutions of the various sera and EDTA-plasma from mouse ($\blacktriangle$) and human ($\blacksquare$) samples on the phosphorylation of IGF-1R. Dose-response curves were assayed three times on three different days and three different western blot analyses, and effects of a sample dilution on the assay were tested for one sample, respectively. Serum and EDTA-plasma were found to phosphorylate IGF-1R in a dose-dependent manner. IGF bioactivity for 10 ng/ml of recombinant IGF-I measured in our assay was set to 1.0 for both mouse and human samples, and the result obtained from each measurement is indicated as the relative phosphorylation level of IGF-1R. Key: IGF-1R, insulin-like growth factor type 1 receptor.

A Mouse serum



B Mouse EDTA-plasma

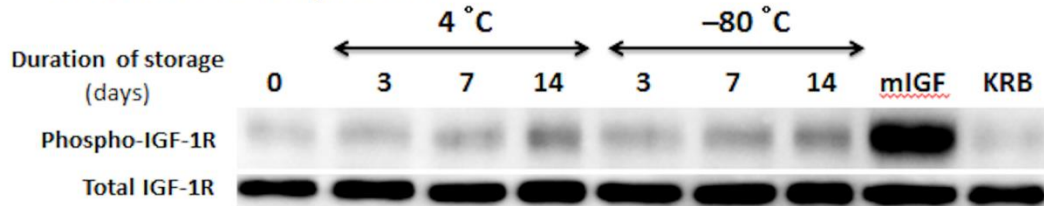
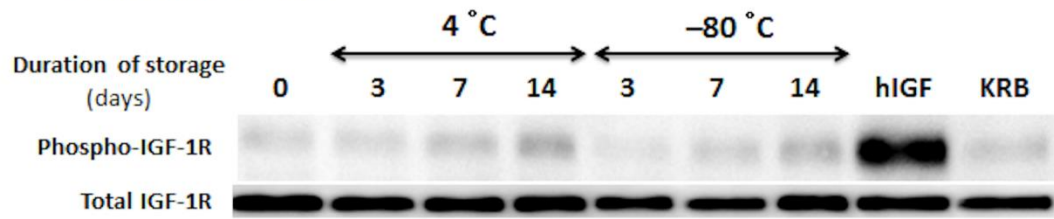


Fig. 2. Representative results using a modified KIRA assay to assess the effects of storage at two different temperatures on mouse bioactive IGFs in serum (A) and EDTA-plasma (B) (from 8-week-old male mouse). The amount of bioactive serum IGF increased markedly in a time-dependent manner irrespective of storage temperature. In EDTA-plasma samples stored at 4 °C, a similar pattern was observed as that observed in serum, but the time-dependent increase in bioactive IGF level was less than in the serum samples. The level of EDTA-plasma bioactive IGFs was nearly stable over 14 days in samples stored at -80 °C. Key: mIGF, recombinant mouse insulin-like growth factor-1 as a positive control; KRB, Krebs-Ringer bicarbonate buffer as a negative control; phospho-IGF-1R, phosphorylated insulin-like growth factor type 1 receptor.

A Human serum



B Human EDTA-plasma

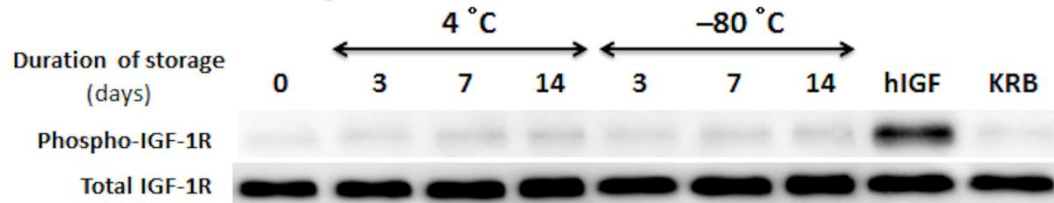


Fig. 3. Representative results using a modified KIRA assay to assess the effects of storage at two different temperatures on human bioactive IGFs in serum (A) and EDTA-plasma (B) (from a 38-year-old man). The detected levels of both serum and EDTA-plasma bioactive IGF increased slightly with time in the samples stored at 4 °C, as seen also in the mouse, but the increases were much smaller. The amounts of bioactive IGF in both serum and EDTA-plasma were nearly stable over 14 days in samples stored at -80 °C. Key: hIGF, recombinant human insulin-like growth factor-1 as a positive control; KRB, Krebs-Ringer bicarbonate buffer as a negative control; phospho-IGF-1R, phosphorylated insulin-like growth factor type 1 receptor.

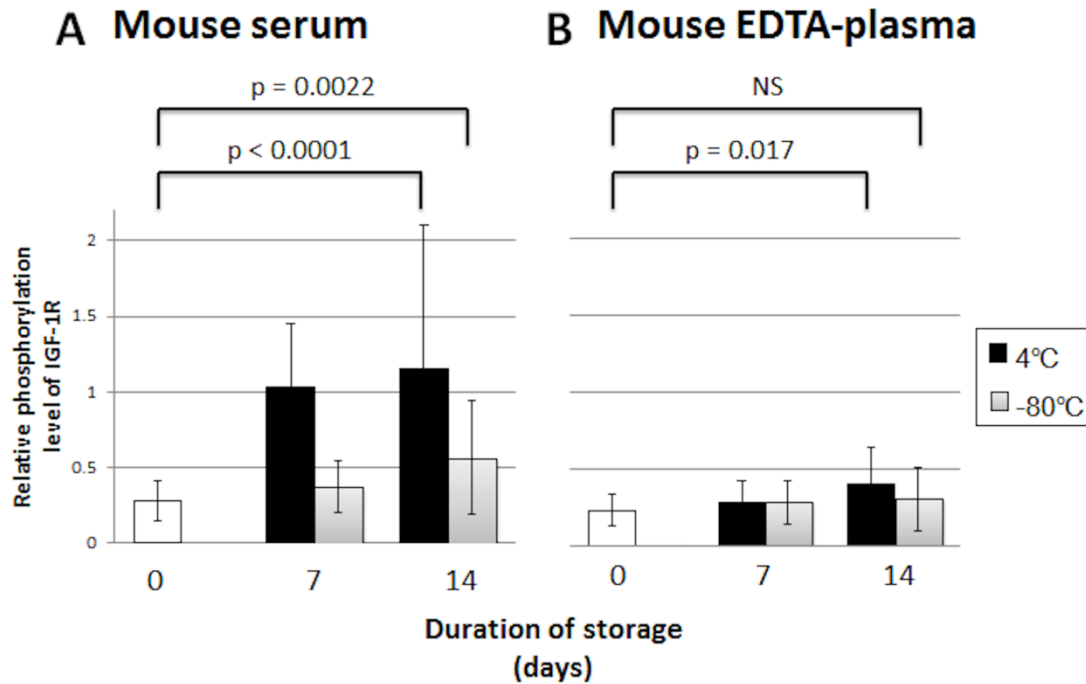


Fig. 4. Effects of storage at two different temperatures (4 °C and -80 °C) on mouse bioactive IGF in serum (A) and EDTA-plasma (B) (n = 21, respectively). Data are expressed as the mean $\pm$ SD for each group. The amount of serum bioactive IGFs increased markedly in a time-dependent manner in samples stored at 4 °C and -80 °C. In EDTA-plasma, a similar pattern to that observed in serum was observed in samples stored at 4 °C, but the time-dependent increase in bioactive IGFs was smaller. EDTA-plasma samples stored at -80 °C were stable over 14 days. The IGF bioactivity for 10 ng/ml of recombinant IGF-I measured in our assay was set to 1.0 for human and mouse samples, and the result obtained from each measurement is indicated as the relative phosphorylation level of IGF-1R. The *p*-values are indicated; NS, not significant.

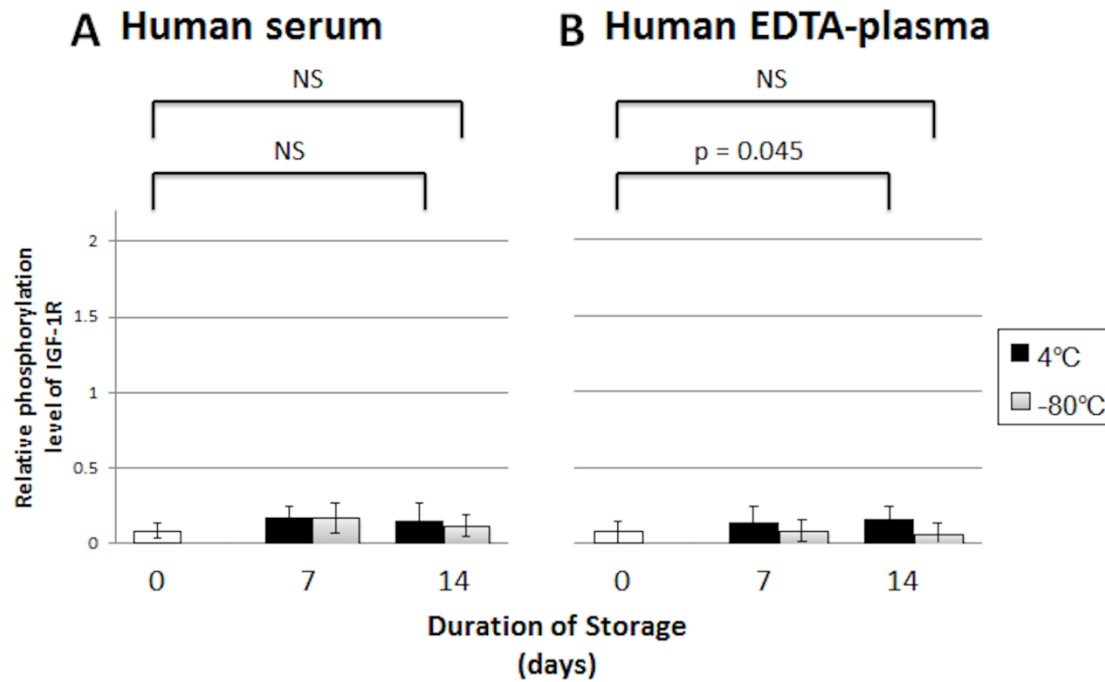


Fig. 5. Effects of storage at two different temperatures (4 °C and –80 °C) on human bioactive IGF in serum (A) and EDTA-plasma (B) (n = 11, respectively). Data are expressed as the mean $\pm$ SD of each group. In the samples stored at 4 °C, the detected levels of both serum and EDTA-plasma bioactive IGFs increased significantly with time, but the increases were much smaller than those observed in mouse samples. The amounts of bioactive IGFs in both serum and EDTA-plasma stored at –80 °C were stable over the 14 days. The storage temperature did not strongly affect the stability of the IGF–IGFBP complex during the sample collection and storage periods. IGF bioactivity for 10 ng/ml of recombinant IGF-I measured in our assay was set to 1.0 for human and mouse samples, and the result obtained from each measurement is indicated as the relative phosphorylation level of IGF-1R. The *p*-values are indicated; NS, not significant.

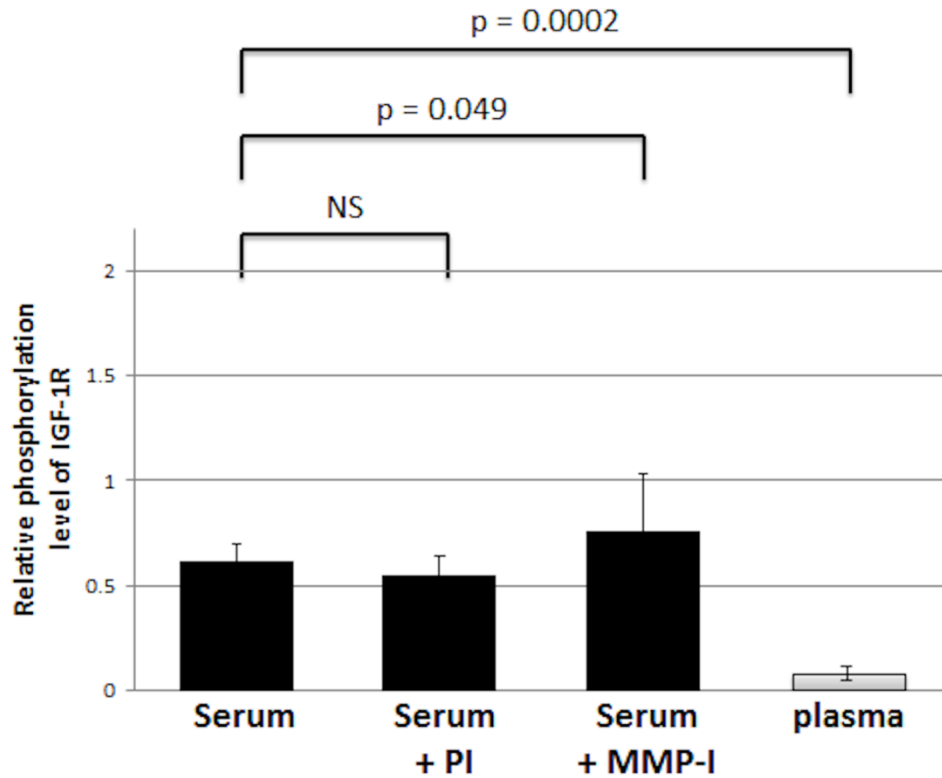


Fig. 6. Effects of protease inhibitors and an MMP inhibitor on mouse IGF bioactivity in samples stored for 7 days at 4 °C (n = 10). Data are expressed as the mean ± SD for each group. The protease inhibitors and MMP inhibitor did not inhibit the increase in mouse IGF bioactivity in samples stored for 7 days at 4 °C compared with EDTA-plasma. IGF bioactivity for 10 ng/ml of recombinant IGF-I measured in our assay was set to 1.0 for human and mouse samples, and the result obtained from each measurement is indicated as the relative phosphorylation level of IGF-1R. The *p*-values are indicated. Key: PI, protease inhibitor cocktail; MMP-I, matrix metalloprotease inhibitor; plasma, EDTA-plasma; NS, not significant.

This manuscript is an accepted manuscript of an article accepted by Elsevier in Growth Hormone & IGF Research on 26 June 2015 available online:
<http://dx.doi.org/10.1016/j.ghir.2015.06.009>