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A SENSITIVE ENZYME IMMUNOASSAY FOR HUMAN BASIC FIBROBLAST GROWTH FACTOR

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SUMMARY: A sensitive sandwich enzyme immunoassay for human basic fibroblast growth factor (HbFGF) was developed employing three monoclonal antibodies (MAb3H3, MAb98 and MAb52). The Fab' fragment of MAb3H3 which inhibits HbFGF biological activity was conjugated to horseradish peroxidase. A mixture of MAb52 and MAb98 was used in the solid phase. Neiter human acidic fibroblast growth factor, hst-1/KS3 product nor acid denatured HbFGF was cross-reactive in this assay system. The detection limit of this assay system was 1 pg/well. Using this assay, some tumor cell lines were revealed to produce a higher level of bFGF than a normal one. Serum samples from normal volunteers were also assayed, and immuno-reactive HbFGF could be detected in 16 out of 57 samples at range 30～206 pg/ml.

Fibroblast growth factors (FGFs) have a potent mitogenic activity for a wide variety of mesoderm- and neuroectoderm-derived cells, and they have a 55 % homology in amino acid sequence. Basic FGF (bFGF) was originally isolated from bovine brain and pituitary, and subsequently purified from various tissues and cell lines. The oncogenic character of FGFs has been pointed out by the highly conserved homology in the FGF family, including int-2, hst-1/KS3 and FGF-5. In mammalian cells, the expression of bFGF cDNA results in the transformation of the host cells. These finding may imply the characteristic of FGF as an active oncogene product including angiogenic activity (reviewed in references [1,2]).

A sensitive assay system for bFGF is therefore important to define the clinical role of bFGF and also for diagnosis of malignant disease in which it participates. Some assay systems for human bFGF (HbFGF) have been developed, using monoclonal antibodies (MAbs) [3] or using polyclonal antibody and heparin [4]. The detection limit of these systems was 3 pg/assay [4]. However the concentration of HbFGF in clinical samples could not be detected by this system because of possible interference by heparin and/or heraran sulfate in the samples.

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In this paper, we describe the construction of a sandwich enzyme immunoassay (EIA) system for HbFGF. This system is suitable for assay of clinical samples and is more sensitive than previously reported assay systems.

MATERIALS AND METHODS

Materials: Anti-HbFGF monoclonal antibodies (MAb52 and MAb98) [3], hybridoma 3H3 which secretes the neutralizing antibody for HbFGF [Hori,et.al., in preparation], recombinant HbFGF (rHbFGF) [5], recombinant HaFGF (rHaFGF) [6] and recombinant hst-1/KS3 gene product [Yoshida,et.al., in preparation] used in the experiments are described elsewhere, respectively. Human melanoma (A375), human lung carcinoma (A549) and human hepatoma (SK-HEP1) cell lines were from the American Type Culture Collection. Human glioma (NMC-G1) was kindly provided by Dr. T. Kondo of the National Medical Center Hospital, Tokyo, Japan. Human umbilical vein endothelial (HUVE) cells were established as described by Kan et.al. [7].

Purification of MAb3H3 and Enzyme labeling: Hybridoma 3H3 cells ($\sim 2 \times 10^6$ cells) were injected intraperitoneally (i.p.) into Balb/c female mice that had received 0.5 ml of mineral oil i.p.. After 7-10 days, ascitic fluid was collected and MAb3H3 was purified by ammonium sulfate precipitation and Mono Q (Pharmacia, Sweden) column chromatography [8]. The Fab' fragment of the purified MAb3H3 (7 mg) was conjugated with horseradish peroxidase (HRP) as described previously [9].

Enzyme immunoassay for HbFGF: Purified MAb52 or MAb98 was dissolved in 0.1 M carbonate buffer (pH 9.6) at a concentration of 10 µg/ml. One hundred microliters of each solution or a mixture of the two were added to each well of a 96-well microtiter plate. After overnight incubation at 4 ℃, the plate was washed with PBS (0.02 M phosphate buffer, pH 7.2, containing 0.15 M NaCl) and each well incubated with 300 µl of Buffer A (PBS containing 25 % Block Ace (Snow Brand Milk Products Co., Japan)) overnight at 4 ℃. After the plate was washed with PBS, 100 µl of standard rHbFGF diluted in Buffer A were added to each well. Following a 24 hour incubation at 4 ℃, the plate was washed with PBS and 100 µl of 3H3-HRP solution in Buffer A were added to each well. After a 2 hour incubation at 25 ℃, the plate was washed, and the bound peroxidase activity was measured with o-phenylenediamine as described previously [10].

To examine the influence of heparin addition in the EIA system, the rHbFGF standard was diluted in Buffer A containing 1, 10 or 100 µg/ml heparin.

For cell extracts and conditioned media, samples and rHbFGF standard were diluted in Buffer B (Buffer A contaning 100 µg/ml heparin).

For serum study, the EIA method was modified as follows. Eighty microliters of the sample sera or "0" serum containing the standard rHbFGF, 80 µl of Buffer B and 80 µl of Buffer C (Buffer A containing 1.5M NaCl, 100 µg/ml heparin and 30 µg/ml mouse IgG) were mixed. The "0" serum was pooled-sera which passed through the MAb98-coupled Sepharose 4B. One hundred microliters of this mixture were added to each well of the MAb52 plus MAb98 coated microtiter plate. Following a 24 hour incubation at 4 ℃, the plate was washed with PBS, and 100 µl of 3H3-HRP solution in Buffer A containing 10 µg/ml mouse IgG were added to each well. After a 2 hour incubation at 25 ℃, the plate was washed, and the bound peroxidase activity was measured.

Acid denatured rHbFGF: One hundred microliters of 200 µg/ml of rHbFGF solution diluted in 20 mM Tris-HCl containing 1 M NaCl (pH 7.4) were added to 900 µl of 1 M acetate buffer (pH 4.0). After various incubation times at 25 ℃, a 200 µl aliquot of the sample was taken and added to 400 µl of 1 M Tris.

Bioassay: Growth stimulation of mouse Balb/c 3T3 cells was assayed as described previously [11]. Briefly, cells suspended in Dulbeco's modified Eagle's medium (DMEM) supplemented with 5 % calf serum (CS) were plated on 96-well microculture plates (2×10^3 cells/well). After overnight incubation, the

culture medium was replaced with 0.2 ml DMEM supplemented with 0.5 % CS. Twenty hours after the addition of 10 µl acid denatured rHbFGF serially diluted in DMEM, ^{3}H-thymidine (37 kBq) was added to each well. After 6 hours, the cells were harvested by trypsinization, and the radioactivity of incorporated ^{3}H-thymidine was measured.

Cell extracts: Cell extracts were prepared as described by Sakaguchi,et.al. [12]. Each cell line was cultured in a culture dish (10 cm in diameter) that contained 15 ml of medium. For A375, A549, SK-HEP1 and NMC-G1 cell lines, GIT medium (Nihon Parmaceutical Co. Ltd., Japan) supplemented with 5 % fetal calf serum (FCS) was used. For HUVE, GIT medium containing 2 ng/ml of rHbFGF and 2.5 % FCS was used. After the cells became 80 % confluent the culture supernatants were harvested. The cells ($2-6x10^{7}$ cells) were then detached with a cell-scrapper. After washing twice with PBS, they were suspended in 0.6 ml PBS and then lysed by sonication for 25 seconds at 0 ℃. After centrifugation at 15,000 rpm for 15 min at 4 ℃, the supernatants (0.7 ml each) were collected.

RESULTS AND DISCUSSION

Recently we reported monoclonal antibodies (MAb12, MAb52, MAb78, MAb98) against rHbFGF and a 2-site sandwich EIA system using MAb52 and MAb78 [3]. The sensitivity of this method was not very high (0.5 ng/assay), and a more sensitive system was reported by Sato et.al. [4]. Furthermore, the sites in the HbFGF molecule recognized by MAb12 and MAb78 were located within the 1-9 amino acid sequence, and those of MAb52 and MAb98 were within the 14-40 amino acid sequence. Therefore our EIA system could not detect a truncated form which lacked the HbFGF amino-terminus. From bovine kidney or corpus luteum, only a truncated form of bFGF which lacked 15 amino acids of the amino-terminus and which retained biological activity was purified [13]. In view of this fact, the truncated form might be present in human tissues. Thereafter, we obtained another monoclonal antibody (MAb3H3) which neutralized the biological activity of HbFGF [Hori,et.al., in preparation]. From these we tried to construct a more sensitive assay system for HbFGF which could be specific for the biologically active form of HbFGF from clinical samples.

In the first experiment, MAb52, MAb98 or the mixture of the two was used to coat wells of 96-well microtiter plates, and the Fab' fragment prepared from MAb3H3 was labeled with HRP and compared in each system. As shown in Fig. 1 the most sensitive result was obtained using the plate coated with the mixture. It was reported that a mixture of 2 MAbs which recognized different epitopes of the same antigen could result in higher affinity compared to that of each MAb [14]. The present result indicated that MAb52 and MAb98 recognize different epitopes of HbFGF.

Since HaFGF and the hst-1/KS3 gene product share a highly conserved homology with HbFGF, it is important to look for cross-reactivity with HaFGF and with the hst-1/KS3 gene product in the EIA system. The results of such an experiment are shown in Fig. 2. Even at the high concentration of 5 ng/ml, both antigens displayed no reactivity (i.e. less than 0.05%). Because MAb3H3 neutralizes HbFGF biological activity, the EIA method was further tested to

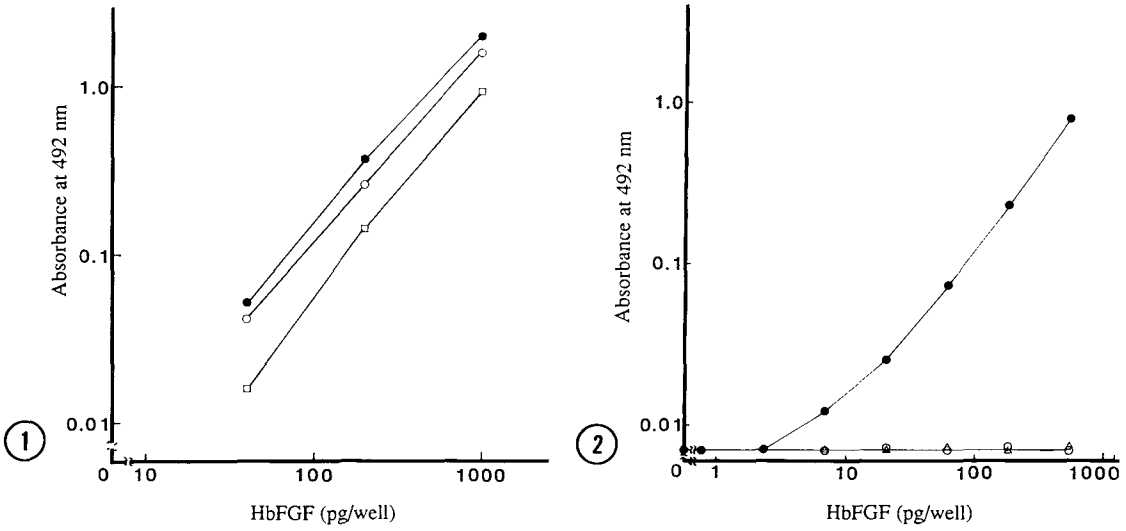

Fig. 1. **Comparison of various sandwich EIAs for rHbFGF.** One hundred microliters of serially diluted rHbFGF was added to each well of a microtiter plate coated with MAb52 (□), MAb98 (○) or a mixture of the two MAbs (●), and incubated overnight at 4℃. After the plate was washed, 100 µl of 200-fold diluted 3H3-HRP was added and the bound peroxidase activities were measured with o-phenylenediamine.

Fig. 2. **Cross-reactivity for rHbFGF, rHaFGF and hst-1 gene product.** One hundred microliters of serially diluted rHbFGF (●), rHaFGF (○) or hst-1 gene product (△) was added to each well of a microliter plate coated with the mixture of MAb52 and MAb98, and incubated overnight at 4℃. After the plate was washed, 100 µl of 200-fold diluted 3H3-HRP was added and the bound peroxidase activities were measured with o-phenylenediamine.

determine whether it reacts only with the biologically active molecule. For this purpose rHbFGF was exposed to pH 4.0 for various times and then tested for biological activity and quantitated by EIA. As shown in Table 1, the biological effect on BALB/c 3T3 cells and reactivity in the EIA decreased at the same level in proportion to the time of acidification. These results indicate that the EIA was specific for bFGF having biological activity.

Table 1. **Reactivity of acid denatured rHbFGF: Comparison of the bio-assay and EIA**

Time (min.)	relative activity (%)	
	bio-assay[a]	EIA[b]
0	100	100
1	57	50
3	20	20
10	0.9	1

[a] Biological activity was measured by a thymidine incorporation assay using Balb/c 3T3 cells. The relative activity is shown as a percentage, with that at time zero being 100.

[b] One hundred microliters of serially diluted acid denatured rHbFGF was added to each well of a microliter plate coated with the mixture of MAb52 and MAb98 and incubated overnight at 4 ℃. After the plate was washed, 100 µl of 200-fold diluted 3H3-HRP was added and the bound peroxidase activities were measured with o-phenylenediamine.

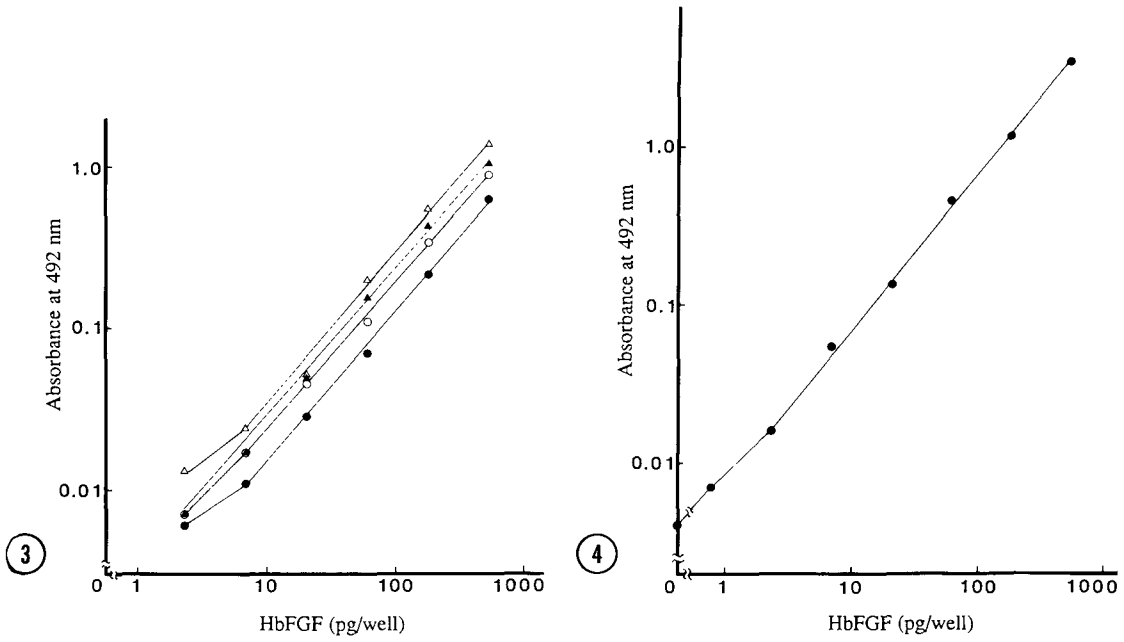

Fig. 3. **Effects of heparin on the rHbFGF sandwich EIA.** One hundred microliters of rHbFGF solution serially diluted in Buffer A containing 1 μg/ml (○), 10 μg/ml (▲), 100 μg/ml (△) or 0 μg/ml heparin (●) was added to each well of a microliter plate coated with the mixture of MAb52 and MAb98, and incubated overnight at 4℃. After the plate was washed, 100 μl of 200-fold diluted 3H3-HRP was added and the bound peroxidase activities were measured with o-phenylenediamine.

Fig. 4. **Typical standard curve for rHbFGF using the sandwich EIA.** One hundred microliters of rHbFGF solution serially diluted in Buffer A containing 100 μg/ml heparin was added to each well of a microliter plate coated with the mixture of MAb52 and MAb98, and incubated overnight at 4 ℃. After the plate was washed, 100 μl of 200-fold diluted 3H3-HRP was added and the bound peroxidase activities were measured with o-phenylenediamine.

Therefore it may be useful for selectively quantitating HbFGF which affects cell proliferation in clinical samples.

Heparin binds to HbFGF with high affinity and is used for the purification of HbFGF. Since heparin and heparan sulfate exist abundantly in blood and organ tissue, HbFGF binds to heparin in them. If the recognition site of MAbs and the heparin binding sites are the same or very close, heparin should act as a competitive inhibitor in the EIA, and cause loss of sensitivity. As shown in Fig. 3, EIA sensitivity was not decreased with heparin. Instead, it increased in proportion to the concentration of heparin. One possible explanation may be that binding of heparin to HbFGF changes the conformation of HbFGF to increase the affinity of HbFGF to the MAbs or to increase stability of HbFGF. A typical standard curve of rHbFGF diluted in Buffer B (Buffer A containing 100 μg/ml of heparin) is shown in Fig. 4. The blank value, which was the optical density at 492 nm in the absence of rHbFGF, was 0.004 ± 0.0013 (mean ± SD, n=6). The detection limit, defined as Blank + 3SD

Table 2. Immuno-reactive bFGF in cell extracts and conditioned media

cell lines		immuno-reactive bFGF	
		cell extract[a] (ng/10^6cells)	conditioned medium (pg/10^6cells)
A375	(melanoma)	1.58	5.3
A549	(lung ca.)	3.29	10.8
SK-Hep1	(hepatoma)	9.63	18.0
NMC-G1	(glioma)	2.01	<3.8
HUVE		0.14	N.D.[b]

[a] Cells (2~6X10^7cells) were detached with a cell scrapper and washed twice with PBS, followed by suspension in 0.6ml PBS and lysis by sonication (25seconds, 0°C). After centrifugation (15,000 rpm) for 15min. at 4°C, the supernatants were assayed.
[b] not determined.

(0.078), was 1 pg/assay. This appears to be better sensitivity than that of previously reported HbFGF assays.

Using the EIA, 10 to 90 times more immuno-reactive HbFGF (ir-bFGF) could be detected in hepatoma (SK-HEP1), melanoma (A375), lung carcinoma (A549) and glioma (NMC-G1) cell lines than in the HUVE cell line (Table 2). According to other previous reports [15,16,17], bFGF was purified from tumor cells such as chondrosarcoma, hepatoma, melanoma and glioma. Our results were consistent with these facts and in addition ir-bFGF could be detected in the lung carcinoma. On the other hand only 0.2 to 0.3 % of ir-bFGF could be detected in the conditioned mediums of these tumor cell lines (Table 2). It was suggested that bFGF produced by cells must be stored in the cells [18,19], because the bFGF molecule lacks the signal sequence which is essential for the molecule to secrete it. Therefore a small quantity of ir-bFGF in the conditioned media might not have been secreted and instead be derived from broken cells.

More ir-bFGF could be detected in tumor cell lines than in a normal one, and normal cells need added rHbFGF in their culture medium. It appears that, although the mechanism is unclear, natural ir-bFGF within a tumor cell stimulates the growth of the same cell.

In the serum study, the sensitivity for HbFGF in serum was 30 pg/ml because the serum was diluted 3 times. When rHbFGF was added to four serum samples at final concentration 82 and 740 pg/ml, the recoveries of it were 103.0±15.9% and 102.9±9.5%, respectively. From analysis of 57 normal volunteer sera, immuno-reactive HbFGF was detected in 16 samples at 30~206 pg/ml. We are now investigating whether the ir-bFGF is truly HbFGF and whether an increase in its concentration signifies malignant disease.

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