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ESTABLISHMENT OF MONOCLONAL ANTIBODIES AGAINST HUMAN ACIDIC FIBROBLAST GROWTH FACTOR

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SUMMARY: Four kinds of hybridomas secreting monoclonal antibodies (MAbs) against human acidic fibroblast growth factor (haFGF) were established using recombinant haFGF as an immunogen. The recognition sites of four MAbs designated AF1-52, 81, 114 and 1C10 for the haFGF molecule were examined by binding studies with synthetic polypeptides and with amino-terminal truncated forms of haFGF. These experiments suggested that AF1-52, 114, and 1C10 MAbs recognize epitopes within the 1-5, 44-132 and 6-43 amino acid sequences, respectively. However, the epitope recognized by the AF1-81 MAb could not be determined. The sandwich EIA method constructed with these MAbs was sensitive to 1.5 pg/well of haFGF and had no cross-reactivity with human basic FGF, bovine aFGF or the hst-1 gene product.

Fibroblast growth factors (FGFs) are known to have mitogenic activity with a wide variety of neuroectoderm- and mesoderm-derived cells in vitro including fibroblasts, endothelial cells, myoblasts and glial cells. They also have angiogenic activity in vivo (1). FGFs are separated into two forms by their pI values: an acidic form with a pI of 5.0-7.0 (aFGF) , and a basic one with a pI of 9.6 (bFGF) (1). Although aFGF and bFGF are encoded by distinct genes, they share 55 % amino acid sequence homology (2).

A number of cultured cells derived from malignant tissues were found to synthesize FGFs and FGF mRNAs (3). Recently, new oncogenes which encode FGF-like proteins, hst-1 (4), int-2 (5), FGF-5 (6), FGF-6 (7) were isolated. These proteins share 30-50 % amino acid sequence homology with aFGF and bFGF. These findings have revealed that FGFs might behave as growth factors not only in the course of normal physiological function but also in carcinogenesis. Indeed, it has been reported that they possess transforming activity under some conditions (8,9).

The quantification of FGFs from human tissues and fluids is therefore important for the diagnosis of malignant diseases. EIA methods and specific MAbs against human bFGF have already been established (10,11). However, a MAb and a useful detection method for human aFGF (haFGF) have not been established.

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In this report, we describe the preparation and characterization of MAbs against haFGF and the construction of a sensitive sandwich EIA method for measurement of it using these MAbs.

MATERIALS AND METHODS

Materials: BALB/c mice were purchased from Shizuoka Animal Center (Hamamatsu, Japan). Human bFGF (hbFGF) and hst-1 mutein (amino-terminal region 1-27 aa truncated form of hst-1) used in the experiments are described elsewhere (12,13). Synthetic polypeptides, haFGF[1-9] (amino-terminal peptide of haFGF,1-9 aa) and haFGF[133-140] (carboxyl-terminal peptide of haFGF, 133-140 aa), were provided by Dr. Wakimasu (Tsukuba Research Institute of Takeda Chemical Ind., Tsukuba, Japan). Bovine aFGF (baFGF) was obtained from R&D Systems, Inc. (Minneapolis, MN).

Preparation of haFGF and its truncated forms: haFGF was purified to apparent homogeneity from recombinant E.coli (14). E.coli expression systems for MuteinⅠ (amino-terminal 1-5 aa truncated form of haFGF) and MuteinⅡ (amino-terminal 1-43 aa truncated form of haFGF) were constructed based on the expression system for haFGF (14). MuteinⅠ was purified by heparin affinity HPLC from the bacterial extract (14). MuteinⅡ was solubilized from inclusion bodies with 6 M urea and then purified by Q-Sepharose column chromatography in the presence of 3 M urea.

Immunization: Eight-week-old female BALB/c mice were immunized 3 times at 3-week intervals. They were immunized first subcutaneously (s.c.) with 100 μg of recombinant haFGF emulsified in Freund's complete adjuvant and the 2nd and 3rd time s.c. with the same dose of recombinant haFGF emulsified in Freund's incomplete adjuvant. Following the third immunization, they were intravenously infused with 100 μg of recombinant haFGF in saline 3 days prior to sacrifice.

Fusion procedure: Three days after the final immunization, the spleen cells from immunized mice were mixed with 8-azaguanine-resistant murine myeloma P3-X63-Ag.8U1 (P3U1) cells at a ratio of 5:1 and fused by a modification of the procedure described previously (15). The culture supernatants from the wells, in which the growth of hybrid cells was observed, were assayed for presence of anti-haFGF antibody by an enzyme linked immunosorbent assay (ELISA) method.

ELISA for antibodies to haFGF: Purified recombinant haFGF was dissolved in 0.01 M sodium bicarbonate buffer (pH 8.5) at a concentration of 10 μg/ml, and 100 μl of the solution was added to each well of a 96-well microtiter plate. The plate was incubated overnight at 4℃, and the fluid removed. Residual protein-binding sites in the wells were blocked by adding Buffer A (phosphate buffered saline (PBS, pH 7.2) containing 25 % Block Ace (Snow Brand Milk products Co., Japan)) followed by incubation overnight at 4℃. One hundred microliters of each hybridoma culture supernatant was added to each of the haFGF-coated wells and incubated for 2 hr at 25℃. After washing the plate 5 times with PBS, 100 μl of a 5000-fold diluted solution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Organon Teknika Corp., West Chester, PA) was added to each well. Following a 2 hr incubation at 25℃, the plate was washed 5 times with PBS and 100 μl of peroxidase-substrate (22 mg of *o*-phenylenediamine and 10 μl of hydrogen peroxide in 10 ml of 0.1 M citrate buffer, pH 5.5) was added. After 30 min. of reaction at 25℃, 100 μl of 4 N sulfuric acid was added to the wells, and the absorbance of each well was measured at 492 nm using a microplate reader (MTP-32, CORONA Co. Ltd., JAPAN).

Neutralizing activity of antibody to haFGF: Mitogenic activity of haFGF was monitored using BALB/c 3T3 clone A31-1-1 cells as previously described (8) and the effects of hybridoma supernatant against this activity were investigated. Briefly, 2×10^3 A31-1-1 cells were suspended in 160 μl of Dulbecco's modified Eagle's medium supplemented with 0.5 % calf serum, then 40 μl of a 5-fold serially diluted hybridoma supernatant and 10 μl of a mitogen solution containing 20 pg of haFGF and 10 μg of heparin were added. After the cells were

incubated for 18 hr at 37℃, they were pulse-labeled with [^{3}H]-thymidine (37kBq/well) for 5 hr, and subjected to radioactivity determination.

Antibody production and purification: Each hybridoma was injected intraperitoneally (i.p.) at 2×10^6 cells into BALB/c female mice that had received 0.5 ml of mineral oil i.p.. Ascitic fluids were collected 7-10 days after the injections. MAbs were purified by ammonium sulfate precipitation followed by hydroxyapatite HPLC column chromatography. Purified AF1-52 MAb was conjugated with HRP described previously (10).

Sandwich EIA for haFGF: Each purified MAb was dissolved in 0.01 M sodium bicarbonate buffer (pH 8.5) at a concentration of 10 μg/ml. One hundred microliters of each solution or a mixture of the three were added to each well of a 96-well microtiter plate. One hundred microliters of various concentrations of haFGF diluted with Buffer A was added to each well and incubated overnight at 4℃. The plate was then processed for measurement of AF1-52-HRP conjugate binding activity (added from a 200-fold dilution) as described above under "ELISA for antibodies to haFGF". For serum study, the sandwich EIA method was modified as follows. Sixty microliters of the sera or "0" serum containing the standard haFGF and 180 μl of Buffer B (Buffer A containing 0.36 M NaCl and 13 μg/ml unrelated mouse IgG) were mixed. The "0" serum was pooled-sera which passed through the AF1-114-coupled Sepharose 4B. One hundred microliters of this mixture were added to each well and incubated overnight at 4℃, then the detection of immuno-reactive haFGF was measured.

RESULTS

Hybridoma secreting antibodies against haFGF: Spleen cells from mice showing a high antibody titer to haFGF in their sera were fused with P3U1 cells and seeded into 960 wells of microculture plates. One to 2 weeks after the fusion, the growth of hybridomas was observed in 390 wells. Culture supernatants were harvested from each well and assayed for the presence of haFGF antibody using an ELISA method. Hybridomas from 4 wells that showed high antibody titer to haFGF were cloned by limiting dilution in the presence of thymocytes (5×10^6 cells/ml) from BALB/c mice as feeder cells. The representative clones were selected and their monoclonal antibodies (AF1-52, AF1-81, AF1-114 and 1C10) were used for all studies. The immunoglobulin subclass of AF1-52 and AF1-81 MAbs was γ2bκ, and that of AF1-114 and 1C10 MAbs was γ1κ .

Determination of MAb recognition site: To determine which regions of the haFGF were recognized by the four MAbs, binding of these MAbs to synthetic polypeptides (haFGF[1-9] and haFGF[133-140]) and to amino-terminal truncated forms of haFGF (Mutein I and Mutein II) was examined. The AF1-52 MAb bound to haFGF[1-9] but not to Mutein I (amino-terminal 1-5 aa truncated form). The AF1-114 MAb bound to Mutein II (amino-terminal 1-43 aa truncated form) but not to haFGF[133-140] and the 1C10 MAb bound to Mutein I but not to Mutein II (Table 1). These results indicate that AF1-52, AF1-114 and and 1C10 MAb recognize epitopes within the 1-5, 44-132 and 6-43 amino acid sequences, respectively. The recognition site of AF1-81 MAb could not be determined because it bound only to haFGF but not to the synthetic polypeptides or to amino-terminal truncated forms of haFGF. Next, we examined whether these MAbs can neutralize the mitogenic activity of haFGF to BALB/c 3T3 cells. However, all of these MAbs had no neutralizing activity (data not shown).

Table 1. Binding ability of MAb to various antigens

MAb	Binding ability[a]				
	haFGF	Pep1-9	Pep133-140	Mutein I	Mutein II
AF1-52	+[b]	+	-[c]	-	-
AF1-81	+	-	-	-	-
AF1-114	+	-	-	+	+
1C10	+	-	-	+	-

[a] One hundred microliters of hybridoma supernatant was added to each well coated with antigen: (Pep1-9: amino-terminal peptide of haFGF, 1-9 aa ; Pep133-140: carboxyl-terminal peptide of haFGF, 133-140 aa ; Mutein I : amino-terminal 1-5 aa truncated form of haFGF; Mutein II : amino-terminal 1-43 aa truncated form of haFGF.). The binding of MAb to the well was examined with goat anti-mouse IgG antibody labeled with HRP.
[b] Bound.
[c] Not bound.

Construction of EIA method for haFGF: To examine whether haFGF could be quantified by a sandwich EIA, four kinds of MAbs were purified and used. One of them (AF1-52 MAb) was labeled with HRP. Each of the other MAbs (AF1-81, AF1-114 and 1C10) or the mixture of all three was used to coat wells of 96-well microtiter plates. As shown in Fig.1, the most sensitive result was obtained from wells coated with the mixture. The detection limit using this EIA method was 1.5 pg/well of haFGF.

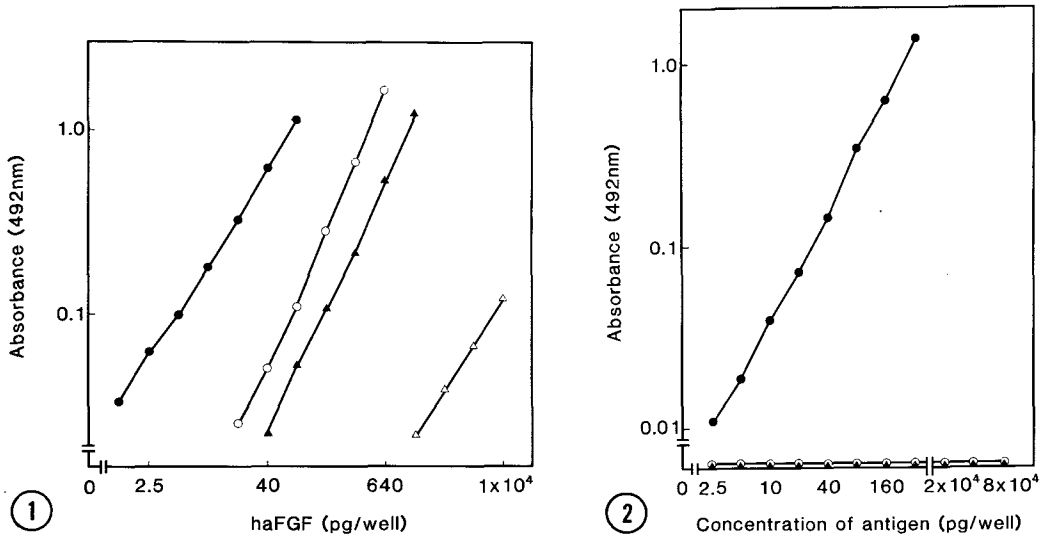

Fig. 1. **Comparison of various sandwich EIAs for haFGF.** One hundred microliters of serially diluted haFGF was added to each well of a microtiter plate coated with AF1-81 (△), AF1-114 (▲), 1C10 (○) or the mixture of these MAbs (●) and incubated overnight at 4 ℃. After the plate was washed, 100 µl of 200-fold diluted AF1-52-HRP conjugate were added and the binding of AF1-52 MAb to each well was examined as described in Materials and Methods.

Fig. 2. **Examination of the specificity of the sandwich EIA.** One hundred microliters of serially diluted haFGF (●), hbFGF (○) or hst-1 mutein (▲) was added to each well of a microtiter plate coated with the mixture of MAbs (AF1-81, AF1-114 and 1C10) and incubated overnight at 4 ℃. After the plate was washed, 100 µl of 200-fold diluted AF1-52-HRP conjugate was added and the binding of AF1-52 MAb to each well was examined as described in Materials and Methods.

Table 2. Effects of heparin on the binding of MAbs to haFGF

Heparin	Binding activity[a]			
(μg/ml)	AF1-52	AF1-81	AF1-114	1C10
0	0.961[b]	0.278	1.498	0.964
10	0.834	0.278	1.478	1.008
100	0.887	0.244	1.398	0.944

[a] Two hundred microliters of each MAb solution and 200 μl of heparin solution were mixed and 100 μl of the mixture was added to the well of the microtiter plate coated with haFGF. The binding of MAb to the well was examined by goat anti-mouse IgG antibody labeled with HRP.
[b] The numbers indicate the mean of duplicate determinations (absorbance at 492 nm).

Specificity of the sandwich EIA: To examine whether the sandwich EIA method specifically recognizes haFGF, the effect of a wide concentration range of hbFGF, hst-1 mutein or baFGF, which share significant structural homology with haFGF, was tested. Fig.2 shows that the sandwich EIA for haFGF could not detect hbFGF or hst-1 mutein even at a high concentration of 800 ng/ml. Additionally, no cross-reactivity (< 0.01 %) could be found with baFGF (data not shown).

Effects of heparin on the binding of MAbs to haFGF: We examined whether heparin inhibits the binding of each MAb to haFGF. The binding of MAbs to haFGF was not inhibited by the presence of 10 or of 100 μg/ml of heparin (Table 2). The effects of heparin on the sandwich EIA for haFGF were also examined. Fig.3 shows that the binding of conjugate (absorbance at 492 nm) was reduced very little in the presence of 10 or of 100 μg/ml of heparin. The detection limit for haFGF in the presence of 100 μg/ml heparin was 2.0 pg/well.

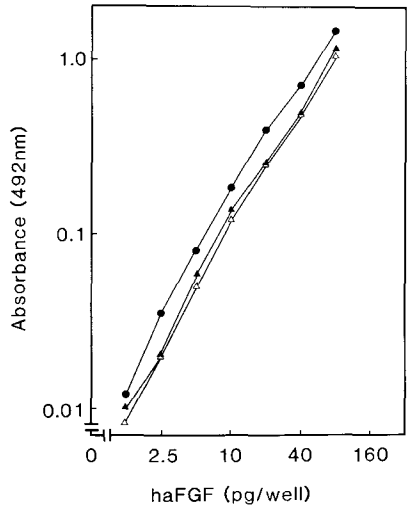

Fig. 3. Effects of heparin on the sandwich EIA for haFGF. Two hundred microliters of serially diluted haFGF and 200 μl of 20 μg/ml of heparin (△), 200 μg/ml of heparin (▲), or PBS containing 1 % BSA (●) were mixed. One hundred microliters of the mixture was added to each well coated with the mixture of three MAbs and incubated overnight at 4℃. After the plate was washed, 100 μl of 200-fold diluted AF1-52-HRP conjugate was added and the binding of AF1-52 MAb to each well was examined as described in Materials and Methods.

DISCUSSION

aFGF has been isolated from brain (16), hypothalamus (17) and retina tissues (18). Glial cell proliferation and neurite extension are both stimulated in vitro by aFGF (1). These observations indicate that aFGF may have an important role in the central nervous system. However, aFGF has recently been found in non-neural tissues such as liver (19), omentum (20) and heart (21). On the other hand, the expression of aFGF in many human glioma cell lines (3) and its transforming activity (9) have been reported. These findings suggest that aFGF might play a role not only in tissue homeostasis but also in carcinogenesis and tumor growth. The true functions of aFGF in normal and pathological tissues are not well understood.

In the present study, we have obtained four kinds of MAbs against haFGF by using recombinant haFGF as an immunogen. The recognition sites of the AF1-52, AF1-114 and 1C10 MAbs were determined to be epitopes within the 1-5, 44-132, and 6-43 amino acid sequences of haFGF, respectively. The AF1-81 MAb bound only to haFGF but not to the synthetic polypeptides or to amino-terminal truncated forms of haFGF. Thus, its recognition site is not clear and it probably recognizes an epitope expressed on the native whole haFGF molecule.

One approach to define the active site of haFGF is to determine the recognition site of MAb that neutralize its growth factor activity. All of the MAbs obtained were found to have no neutralizing activity, so the active site of haFGF could not be defined in this way. However, although the AF1-52 and 1C10 MAbs which recognize the amino-terminal region could not neutralize the biological activity of haFGF, the amino-terminal truncated forms of haFGF (Mutein I and Mutein II) have the same biological activity (14). These results are thus in agreement with an assumption that the carboxyl-terminal, but not the amino-terminal, region of haFGF may be essential for biological activity.

Using four MAbs, several kinds of sandwich EIA methods for haFGF were constructed and compared. The most sensitive system was obtained when the mixture of three MAbs (AF1-81, AF1-114 and 1C10) was used as a solid phase. These MAbs recognize different epitopes of haFGF. Therefore, the mixture of these three antibodies may have worked better due to a synergistic action to trap haFGF on the solid.

Heparin binds to haFGF with high affinity and is used effectively for the purification of haFGF. Heparin also potentiates the biological activity of haFGF (1). Although the precise mechanism is unknown, it is believed that heparin binds to haFGF and changes the tertiary structure to prevent its proteolysis and/or increase its affinity for its receptor (1). Since heparin and heparin-like glycosaminoglycans exist abundantly in blood and organ tissues, aFGF is thought to be associated with them in vivo. The MAbs obtained in the present study were found not to inhibit the binding of heparin to haFGF. Thus the binding site of heparin differs from the recognition sites of MAbs used in the present study. This is presumably the reason why the sandwich EIA of the present study exhibited high sensitivity to haFGF in the presence of a large amount of heparin.

The sandwich EIA method constructed in the present study is highly sensitive in the presence of heparin and shows no cross-reactivity with hst-1 protein, hbFGF or baFGF. Therefore, this method should be useful for the quantitative determination of haFGF in clinical materials. In one preliminary experiment, an immuno-reactive substance could be identified less than 100 pg/ml in 58 normal human sera using the EIA method. We are now investigating whether the immuno-reactive substance is truly haFGF and whether an increase in its concentration may signify malignant diseases.

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