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Elevated Level of Plasma Basic Fibroblast Growth Factor in Multiple Myeloma Correlates with Increased Disease Activity

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Recent reports that bone marrow angiogenesis is increased in multiple myeloma prompted us to examine plasma concentrations of angiogenic growth factors and to elucidate their clinical and biological significance. In 45 cases including 36 cases of multiple myeloma and 9 cases of monoclonal gammopathies of undetermined significance (MGUS), plasma concentrations of basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) were evaluated. FGF-2 was significantly elevated in 25 out of 45 (56%) of the patients with multiple myeloma compared with control subjects (median 9.01 pg/ml vs. 1.58 pg/ml, $P<0.0001$). The 25 cases were all active multiple myeloma, and none of the non-active myeloma and MGUS patients showed a high FGF-2 level. VEGF level was also elevated in 26 out of 45 patients (58%) compared with control subjects (median 42.0 pg/ml vs. 15.8 pg/ml, $P<0.0001$ for VEGF). VEGF concentration was high in 20 active myelomas, but also in one non-active myeloma and five MGUS. Elevation of FGF-2 level was associated with β2-microglobulin level, anemia and bone marrow plasma cell percentage, which represent disease activity. Interestingly, none of five Bence-Jones type myelomas, including four clinically active cases, revealed a high plasma FGF-2 level, while all of them showed a high VEGF level. In all five responders, the plasma FGF-2 levels were significantly decreased after chemotherapy. FGF-2 was immunohistochemically detected in the bone marrow myeloma cells of the patients with high plasma FGF-2 level. We conclude that plasma concentration of FGF-2 can be a useful indicator of disease activity.

Key words: Multiple myeloma — Basic fibroblast growth factor — Angiogenesis — Vascular endothelial growth factor — Disease activity

Multiple myeloma (MM) is one of the major hematologic malignancies, which mainly affects elderly people. In spite of intervention with conventional chemotherapy, the clinical course of the disease is progressive and exclusively fatal.[1,2] Growth factors are considered to play an important role in disease progression of patients with MM, and especially interleukin (IL)-6 has been identified as a crucial cytokine involved in expansion of the tumor clones.[3] Recently, it was also reported that microvessel density (MVD) in the bone marrow of MM was increased, and bone marrow angiogenesis is assumed to play an important role in disease progression and clinical disease activity.[4–6]

Fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) are pleiotropic growth factors that exert growth stimulation of normal and neoplastic cells. FGFs and VEGF are also known as major potent positive regulators of angiogenesis both *in vitro* and *in vivo*.[7] These angiogenic factors have been indicated to play roles in the progression of several types of solid tumor. For example, elevated serum concentration of basic fibroblast growth factor or FGF-2 was reported to be associated with histological grade and stage of renal cell carcinoma.[8] Recently, several reports have analyzed the biological role of FGF-2 and VEGF in hematologic malignancies.[9–11] Aguayo *et al.* reported the elevated plasma concentration of these angiogenic factors and increased bone marrow angiogenesis in acute and chronic leukemias.[12] Vacca *et al.* indicated association of increased bone marrow angiogenesis with disease activity and also showed elevated intracellular accumulation of FGF-2 in myeloma cells.[13] It was also reported that VEGF was immunohistochemically detected in myeloma cells.[14] More recently, simultaneous elevation of serum FGF-2 and VEGF levels was correlated with poor prognosis in non-Hodgkin's lymphoma.[15]

Here we report that the plasma concentrations of FGF-2 and VEGF were frequently elevated in patients with MM. Elevation of FGF-2 level was observed only in clinically

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active MM, but not in non-active MM and monoclonal gammopathies of undetermined significance (MGUS). We also found that FGF-2 is produced by myeloma cells.

MATERIALS AND METHODS

Patients A total of 45 Japanese patients at Keio University Hospital who fulfilled the South West Oncology Group (SWOG) diagnostic criteria for MM (n=36) and MGUS (n=9) were studied.[16] Twenty-six cases were untreated, and the other 19 cases were treated, but blood and bone marrow samplings were conducted more than one month after the previous treatment. The patients were defined as active (n=33) or non-active (n=12), using the criteria of Vacca *et al*.[4] Briefly, active were those: (a) at diagnosis, with symptomatic disease and an increase in the M-component level; (b) at relapse; (c) with rapidly progressive disease, characterized by severe bone pain, hypercalcemia and pancytopenia. Non-active were those in the off-treatment plateau phase of stable M-component levels with no clinical symptoms. All patients gave informed consent for the use of peripheral blood and bone marrow biopsy specimens.

Blood sampling and measurement of plasma growth factors Platelets contain large amounts of FGF-2 and VEGF, and these growth factors may be released during the clotting process and the separation of serum.[17] Therefore, we decided to use plasma rather than serum to evaluate the concentrations. Venous blood samples were collected in tubes containing EDTA and were centrifuged (2000*g*, 10 min) within 2 h after blood sampling and stored at −80°C until assayed. Plasma concentrations of FGF-2 and VEGF were analyzed using a sandwich ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, patients' samples were added to microplates and incubated at room temperature for 2 h. The plates were washed three times, then enzyme-linked polyclonal antibodies were added, and the mixtures were incubated at room temperature for 2 h. After washing, substrate solution was added to develop blue color. Intensity of the color was measured by microplate reader at 450 nm. All samples were assayed in duplicate. In these enzyme immunoassay systems, the detection limit of FGF-2 in plasma was 1 pg/ml and that of VEGF was 15.6 pg/ml. Elevated FGF-2 and VEGF levels were defined as being greater than the highest value in the normal control subject group. This resulted in cut-off values for FGF-2 of 7.67 pg/ml and for VEGF of 38.3 pg/ml, respectively. In five active myelomas, changes of plasma growth factor levels after chemotherapy with vincristine, adriamycin and dexamethasone (VAD) were examined.

Immunohistochemical evaluation of bone marrow angiogenesis and detection of FGF-2 Ten percent of buffered formalin-fixed, paraffin-embedded bone marrow biopsy specimens were examined. All blood vessels were highlighted by staining endothelial cells with an anti-CD34 murine monoclonal antibody at 1:100 dilution (Novocastra, New Castle, UK). Assessment of bone marrow angiogenesis was basically performed according to Perez-Atyde *et al*.[18] Five representative non-overlapping microscopic areas were randomly chosen from each bone marrow biopsy at ×80 magnification, and the mean number of CD34-stained vessel contours per field was obtained. Bone marrow angiogenesis was evaluated by calculating the number of vessels per mm^2. Mean vessel density of 6 patients with non-hematological disorder was 43.5±20.3 (20.4−70.4)/mm^2. Immunohistochemical staining of FGF-2 was also performed using an anti-human FGF-2 rabbit polyclonal antibody at 1:100 dilution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Cell lines Three human myeloma cell lines (HS-Sultan, KMS12-BM, RPMI8226) were obtained from Japanese Cancer Research Resources Bank (JCRB, Tokyo), and the U266 line was from the American Tissue Culture Collection. All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) at 37°C in a humidified atmosphere with 5% CO_2.

Measurement of FGF-2 in the conditioned medium Cells (5×10^5) were incubated with 1 ml of RPMI-1640 medium supplemented with 10% dialyzed fetal bovine serum (GIBCO Laboratories, Grand Island, NY) for 48 h in 24-well plates (Corning, Inc., Corning, NY). The conditioned medium was analyzed for FGF-2 using a sandwich ELISA technique (R&D Systems). Each value was calculated by averaging results for triplicate samples.

Reverse transcriptase-polymerase chain reaction (RT-PCR) One microgram of total cellular RNAs were used for cDNA synthesis using reverse transcriptase (Super Script, Life Technologies) under the conditions recommended by the manufacturer. The primer sequences used for FGF-2 amplification were as follows. P1: (sense) CAAGCGGCTGTACTGCAA, and P2: (anti-sense) CCTTCATAGCCAGGTAACGG. PCR reaction was performed using recombinant *Thermus aquaticus* DNA polymerase (Toyobo Co., Tokyo). Reaction conditions were 35 cycles of denaturation (94°C for 15 s), annealing (58°C for 15 s) and extension (72°C for 30 s). As an internal control, β-actin cDNA was also amplified simultaneously using a specific primer set (Toyobo). All products were separated on 1.5% agarose gel.

Western blotting Cell pellets of myeloma cell line were lysed in the sample buffer (62.5 m*M* Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 80 m*M* dithiothreitol, 0.01% bromophenol blue). Lysates (50 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA). The

membrane was probed with rabbit antihuman-FGF-2 polyclonal antibody (Santa Cruz) diluted 1:200 with phosphate-buffered saline (PBS)/0.1% Tween 20. Detection was performed using the enhanced chemiluminescence system ECL-plus (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions.

Statistical analyses For evaluation of plasma concentration of FGF-2 and VEGF, the Mann-Whitney *U* test was used. Association of elevated growth factor level with clinical parameters was evaluated by the Fisher exact test and by the χ^2 test. Effects of treatment on growth factor concentrations were evaluated by the Wilcoxon test. The software package Stat-View 5.0 (SAS Institute, Inc., Cary, NC) was used for statistical analysis. Multivariate analysis for evaluation of correlations between FGF-2 level and clinical parameters, including age, sex, VEGF level, stage, disease activity, hemoglobin level, β2M, bone marrow plasma cell percentage and serum calcium level was performed by logistic regression analysis using the SAS package. *P* values <0.05 were considered as statistically significant.

RESULTS

Elevated plasma concentration of angiogenic growth factors in multiple myeloma Plasma concentrations of FGF-2 and VEGF were examined by ELISA in 45 MM and MGUS patients (Fig. 1). Median values of FGF-2 and VEGF levels are also shown in Fig. 1. Plasma concentration of FGF-2 in cases was significantly higher than that of normal control subjects ($P<0.0001$). The FGF-2 level of clinically active MM was significantly higher than that of the control group ($P<0.0001$) and that of non-active MM and MGUS ($P=0.0003$). There was no statistically significant difference of FGF-2 concentrations between non-active cases, including non-active MM and MGUS, and the control group ($P=0.18$). Plasma VEGF concentrations were also elevated in all cases compared with the control group ($P<0.0001$). The VEGF level in active MM was significantly higher than that of healthy control subjects ($P<0.0001$), but there was no statistically significant difference between active and non-active cases ($P=0.086$). The VEGF level of non-active MM and MGUS was significantly higher than that of the control group ($P=0.015$).

Correlation between FGF-2 level and clinical parameters To investigate the clinical significance of the elevated plasma FGF-2 level, the relationship between FGF-2 concentration and clinical parameters was analyzed (Table I). When the cut-off value was taken as 7.67 pg/ml, which is the highest value in the control group, plasma FGF-2 concentration was elevated in 25 of 45 cases (57%), in 25 of 33 active MM patients (76%) and none of 12 non-active MM and MGUS patients. The correlation of the plasma FGF-2 level with disease activity was statistically significant ($P<0.0001$). Therefore, clinical evaluation using 7.67 pg/ml as the cut-off value well represents disease activity, and it was decided to use this cut-off value for further analyses. MGUS, anemia, β2M, immunoglobulin (Ig) subclass and bone marrow plasma cell percentage were also statistically significant factors in all cases. In 33 cases with active MM, anemia, β2M and bone marrow plasma cell percentage were not statistically significant factors. Therefore, these factors were considered to relate to disease activity. Multivariate analysis by using a logistic regression model also indicated that disease activity was an independent factor correlating with plasma FGF-2 level ($P=0.0013$). Interestingly, plasma FGF-2 level was within

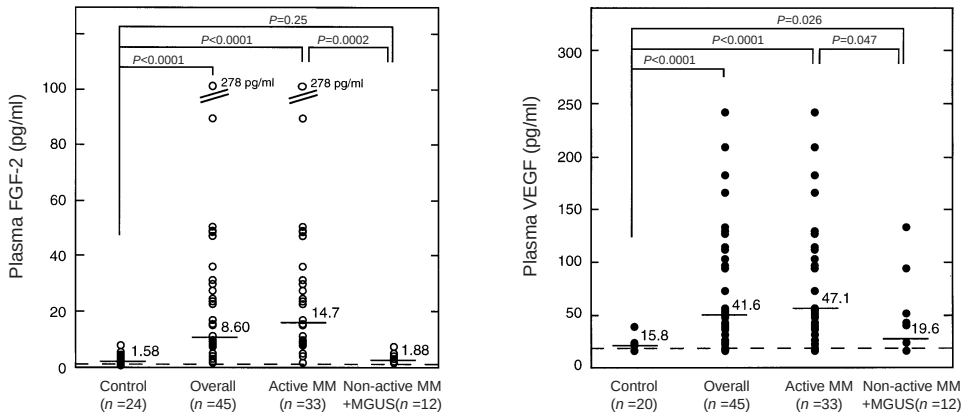

Fig. 1. Plasma concentrations of FGF-2 and VEGF in the patients with MM and MGUS. The dashed line indicates the limit of detection of the immunoassay. The bar and numbers illustrate the median for each group. The Mann-Whitney test was used to compare plasma FGF-2 and VEGF concentrations in different groups.

Table I. Correlation between Plasma FGF-2 Concentration and Clinical Parameters

Parameters		Total No.	High FGF-2	Low FGF-2	*P* value[b]
Overall		45	25	20	
Age	60 yr≤	17	11	6	0.37
	60 yr>	28	14	14	
Gender	male	25	12	13	0.37
	female	19	13	7	
MGUS or multiple myeloma	MGUS	9	0	9	0.0002
	myeloma	36	25	11	
Durie-Salmon stage	I+II	10	5	5	0.22
	III	26	20	6	
Disease activity[a]	active	33	25	8	>0.0001
	non-active	12	0	12	
Anemia	Hb<10 g/dl	20	16	4	0.006
	Hb≥10 g/dl	25	9	16	
Serum creatinine	>2.0 mg/dl	6	4	2	0.68
	≤2.0 mg/dl	39	21	18	
β_2-microglobulin	2.3 mg/liter<	29	21	8	0.001
	2.3 mg/liter≥	15	3	12	
Serum calcium	9.8 mg/dl<	11	9	2	0.086
	9.8 mg/dl≥	32	16	16	
C-reactive protein	0.15 mg/liter<	19	12	7	>0.99
	0.15 mg/liter≥	21	13	8	
Immunoglobulin subclass	IgG	28	17	11	0.03
	IgA	7	6	1	
	IgD	4	2	2	
	Bence-Jones protein	5	0	5	
	IgM	1	0	1	
Light chain	κ	27	12	15	0.06
	λ	18	13	5	
Bone marrow plasma cell	10%≤	26	20	6	0.004
	10%>	17	5	12	
Bone marrow micro vessel density	120/mm^2≤	10	7	3	>0.99
	120/mm^2>	10	7	3	
Active multiple myeloma		33	25	8	
Durie-Salmon stage	I+II	7	5	2	>0.99
	III	26	20	6	
Anemia	Hb<10 g/dl	20	16	4	0.68
	Hb≥10 g/dl	13	9	4	
β_2-microglobulin	2.3 mg/liter<	27	21	6	0.58
	2.3 mg/liter≥	5	3	2	
Immunoglobulin subclass	IgG	19	17	2	0.002
	IgA	6	5	1	
	IgD	4	3	1	
	Bence-Jones protein	4	0	4	
Light chain	κ	17	12	5	0.69
	λ	16	13	3	
Bone marrow plasma cell	10%≤	25	20	5	0.63
	10%>	7	5	2	

a) Vacca's criteria. See "Materials and Methods."
b) The χ^2 test was used for immunoglobulin subclass, and the Fisher exact test was used for other clinical parameters.

the normal range in all of the Bence-Jones protein (BJP)-type MM.

Plasma VEGF concentration was also elevated in 26 out of 45 of the patients (58%), in 20 out of 33 active MM (60.6%) and in 6 of 12 non-active MM and MGUS (50%). No statistically significant correlation was seen with any of the clinical parameters using the Fisher exact test and the χ^2 test (data not shown). As mentioned earlier, elevation of plasma VEGF level likely occurs in the very early stages of myelomagenesis, and therefore it is not surprising that no significant difference was seen among MM patients. Contrary to the case of FGF-2, all of the patients with BJP-type MM showed elevated VEGF. Therefore, VEGF may be a key factor that regulates the progression of BJP-type myeloma cells.

Change of plasma growth factor concentrations of five responders after chemotherapy is shown in Fig. 2. FGF-2 level was clearly decreased in all five responders after chemotherapy. However, the VEGF level was not significantly decreased after treatment.

An increased activity of angiogenesis in the bone marrow of the patients with MM has been reported (Refs. 6 and 18 and Wenlin *et al.*, in preparation). It is speculated that up-regulation of plasma growth factor concentration may lead to increased marrow angiogenesis. In our study, bone marrow biopsy specimens from twenty patients were available. As shown in Table I, however, there is no significant relationship between plasma FGF-2 level and MVD. No correlation of plasma VEGF level with bone marrow MVDs was observed, either (data not shown).

Production of FGF-2 by myeloma cells The next important question is, which cells produce FGF-2? It is especially of interest whether myeloma cells produce FGF-2. Expression of FGF-2-gene product was examined using four multiple myeloma lines. As shown in Fig. 3A, FGF-2 transcripts were detected in three myeloma cell lines, U266, KMS12-BM and RPMI8226 cells, by RT-PCR analysis. Immunoblot analysis also revealed a 22.5-kDa band in U266 cells. FGF-2 does not contain a signal peptide sequence, and the mechanism of excretion of FGF-2 from the cell is still unclear. To elucidate whether FGF-2 produced by myeloma cells is secreted, the concentration of FGF-2 in the culture medium was also examined. FGF-2 was detected in the conditioned medium of U266 cells, but it was below the limit of detection in the medium of HS-Sultan cells, which do not produce FGF-2 (Fig. 3, A and B). To examine production of FGF-2 by primary myeloma cells, FGF-2 was immunohistochemically stained using bone marrow biopsy specimens. Representative cases are shown in Fig. 4, in which FGF-2 was detected in the myeloma cells of the patients with high plasma FGF-2 level (Fig. 4c). There was no significant staining in this case using non-immune rabbit serum (data not shown). When the bone marrow specimens of active MM patients with low FGF-2 level were also examined, no significant staining of FGF-2 was detected in myeloma cells (Fig. 4d).

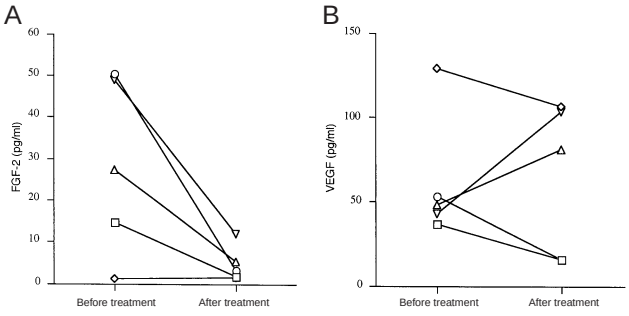

Fig. 2. Plasma FGF-2 and VEGF in serial samples from five responding patients. Plasma concentrations before and after chemotherapy are shown. Median pre-treatment and post-treatment levels of FGF-2 were 27.1 pg/ml and 3.19 pg/ml, respectively (P=0.080 by the Wilcoxon test). Those of VEGF were 48.0 pg/ml and 80.6 pg/ml, respectively (P=0.89 by the Wilcoxon test).

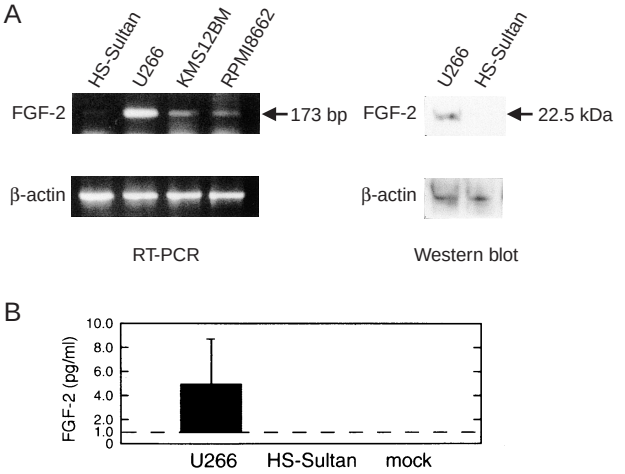

Fig. 3. Expression and secretion of FGF-2 by myeloma cells. A. Transcripts of FGF-2 and the protein were detected in myeloma lines, by RT-PCR and western blotting, respectively. As an internal control, β-actin was used for RT-PCR. B. FGF-2 protein was detected in the culture medium of U266 cells by ELISA methods. Mean value±SD of triplicate samples is shown. Mock indicates cell-free culture medium. Dashed line indicates the detection limit of the immunoassay.

DISCUSSION

Recent advances in genetic as well as biological analyses have revealed that multiple myeloma evolves via a multistep process involving alterations in various onco-

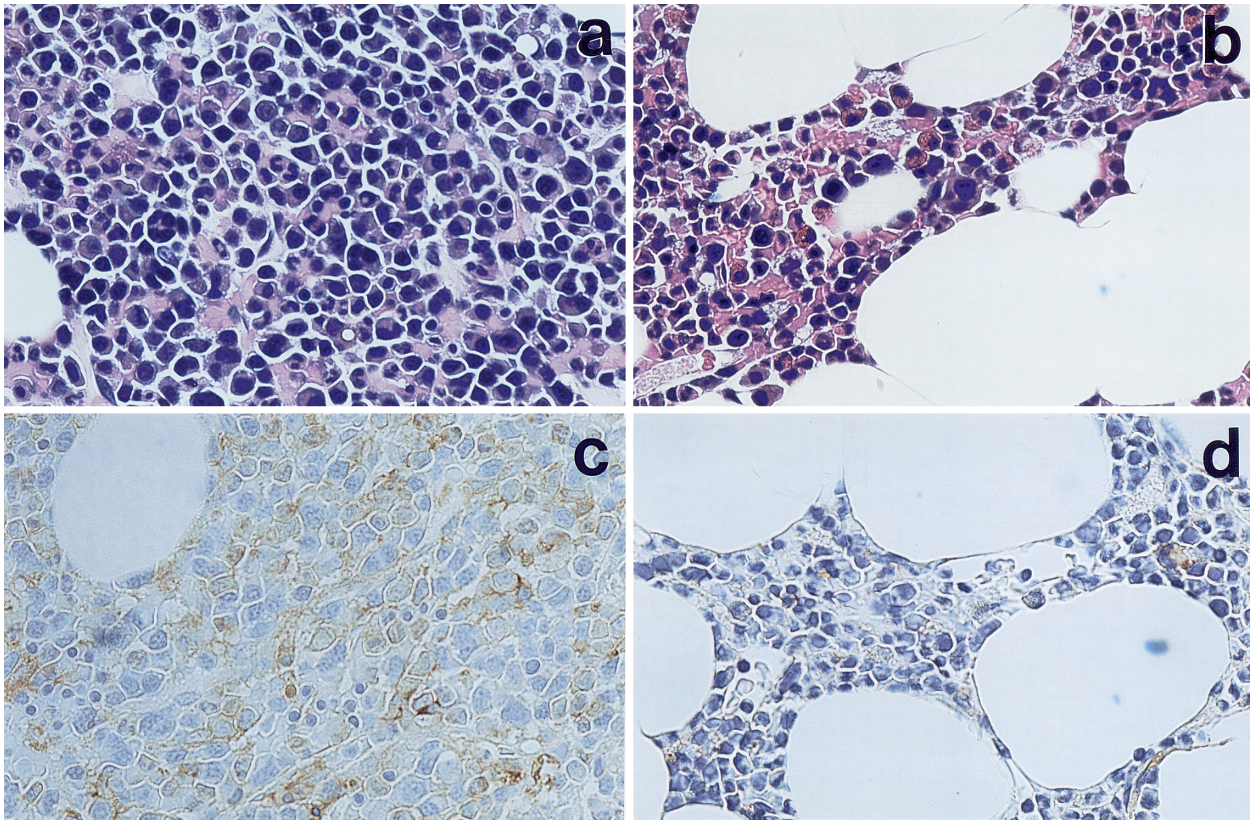

Fig. 4. Immunohistochemical detection of FGF-2 in the bone marrow of active myeloma cases. H&E staining (a, b) and immunohistochemical staining for FGF-2 (c, d) of the two representative positive and negative cases were shown. (a, c) An IgA-type active myeloma patient who revealed high plasma FGF-2 level (278 pg/ml). Myeloma cells were positively stained with anti-human FGF-2 polyclonal antibody. (b, d) A Bence-Jones-type active myeloma whose plasma concentration of FGF-2 was 1 pg/ml. There was no significant staining of myeloma cells.

genes and tumor suppressor genes. Cytokines and growth factors also play important roles in myeloma cell growth in an autocrine or a paracrine manner.[19] Recently, it has also been reported that bone marrow MVD significantly increases in active MMs.[4–6, 20] Thus, we examined the plasma concentrations of angiogenic growth factors, and found significant elevations of FGF-2 and VEGF in 56–58% of active MMs. As shown in Fig. 1, elevation of FGF-2 level is observed specifically in active MM, while increase of VEGF concentration occurs not only in active MM, but also in non-active MM and MGUS. Therefore, it is speculated that FGF-2 may predominantly increase at the transition from non-active to active disease, while VEGF may be elevated in the earlier clinical course, such as MGUS or non-active MM. As shown in Table I, elevation of FGF-2 concentration was associated with advanced anemia, high B2M level and high plasma cell percentage in the bone marrow, which also indicate disease activity. In addition, multivariate analysis showed the association of disease activity with elevated FGF-2 level. As shown in Fig. 2, plasma FGF-2 levels were significantly decreased after chemotherapy in responders. This fact also indicates the association of FGF-2 levels with disease activity. In our study, FGF-2 level was low in all of the BJP-type MMs (Table I). Although the reason is unclear, the association between low FGF-2 level and BJP-type does not relate to disease activity, because the FGF-2 level was low even in all four clinically active BJP-type MMs.

The exact role of FGF-2 in the progression of MM is not clear. To elucidate the effect of FGF-2 on bone marrow angiogenesis, the relationship between plasma growth factor level and MVD in the bone marrow was examined (Table I). However, we could not find a significant correlation. In addition, the MVD of two active MMs was within the normal range, despite their high plasma FGF-2 level (data not shown). It was also reported that the patients with chronic lymphocytic leukemia show a high plasma FGF-2 level, but their bone marrow MVD is com-

parable with that of control subjects.[12] These results suggested that elevated plasma FGF-2 may contribute to the progression of B-cell malignancies not only by increasing bone marrow angiogenesis, but also via some other unknown mechanism. A recent report showed that 20–30% of MM patients carry chromosomal translocations of the immunoglobulin heavy-chain (IgH) locus on chromosome 14q32 with FGF receptor-3 (fgfr3) on 4p16 locus. Some of the translocated fgfr3 have activating mutations within their coding sequence.[21] Furthermore, active alleles of fgfr3 have transforming potential to NIH3T3 cells and plasma cells.[21, 22] These data provide a strong link between FGF signaling and the pathogenesis of MM. We have previously reported the expression of the gene for FGF receptor-1, a high-affinity receptor for FGF-2, in myeloma cell lines.[23] Activation of FGF receptors on myeloma cells may be important for progression of myeloma cells.

The origin of FGF-2 is of great interest. FGF-2 is ubiquitously expressed in various tissues, especially in mesenchymal cells. The concentration of FGF-2 is much higher in bone marrow than in peripheral blood.[24] Therefore, it is speculated that FGF-2 is released from bone marrow interstitial cells or bone matrix.[20] In our study, FGF-2-gene products were also detected in three myeloma cell lines and also in bone marrow biopsy specimens. Otsuki *et al.* reported the expression of FGF-2 transcripts in all six cell lines which they established.[25] Recently, it has been reported that myeloma cells produce VEGF and hepatocyte growth factor (HGF).[14, 26] Therefore, myeloma cells *per se* are considered to be one of the sources for these growth factors.

In conclusion, we found that plasma level of FGF-2 is significantly elevated in clinically active MMs, and this should be a good indicator for disease activity.

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