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Original Article

Tumor angiogenesis in the bone marrow of multiple myeloma patients and its alteration by thalidomide treatment

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Angiogenesis in solid tumors is important to tumor growth, invasion and metastasis. Recently, it has been suggested that angiogenesis plays a certain role in the development of hematopoietic malignancies, including leukemia and multiple myeloma. We evaluated tumor angiogenesis in the bone marrow (BM) of multiple myeloma (MM) patients by calculating microvessel density (MVD) in needle-biopsy specimens obtained from 51 cases of untreated MM or monoclonal gammopathy of undetermined significance (MGUS). The MVD in the BM of donors for transplantation and patients with non-hematological diseases was calculated as a control. There was an obvious increase in MVD in the BM of MM patients, and the MVD correlated with the grade of myeloma cell invasion of the BM in the untreated MM cases. It was recently reported that thalidomide might be effective for the treatment of MM. We assessed the effect of thalidomide on angiogenesis in BM treatment of 11 patients with refractory MM. The concentration of M-protein in the serum or urine of seven of the 11 patients was reduced by at least 30% after thalidomide treatment, and MVD in the BM decreased in three of these seven cases in response to thalidomide. Increased plasma concentrations of basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) were observed in all 11 cases before thalidomide administration and both levels were reduced after treatment with thalidomide. Augmented angiogenesis in the bone marrow of MM patients was confirmed in the present study. It seems that thalidomide is effective in the treatment of MM through the impairment of angiogenesis by decreasing FGF-2 and VEGF production. This is the first report on pathological evidence in the bone marrow of MM before and after thalidomide treatment, in Japan.

Key words: angiogenesis, CD34, fibroblast growth factor, multiple myeloma, thalidomide, vascular endothelial growth factor

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Multiple myeloma (MM) is a plasma cell neoplasm that originates in the bone marrow and involves the entire skeleton. The myeloma cells synthesize large amounts of complete and/or incomplete immunoglobulins, which may cause a hyperviscosity syndrome, amyloidosis, and renal failure. Multiple myeloma accounts for approximately 10% of all hematological malignancies. Chemotherapy with melphalan and/or dexamethasone is effective in some cases but relapses often occur, and relapsed MM might become refractory to conventional chemotherapy.[1]

Angiogenesis is indispensable to growth, invasion and metastasis by solid tumors and occurs in other diseases, including rheumatoid arthritis, psoriasis, scleroderma and diabetic retinopathy.[2–4] Angiogenesis has been postulated to be regulated by a balance between certain angiogenic and antiangiogenic factors. Angiogenic factors include soluble factors, such as vascular endothelial growth factor (VEGF), acid and basic fibroblast growth factor (aFGF and bFGF or FGF-2), angiopoietin-1, hepatocyte growth factor (HGF) and interleukin-8 (IL-8), and adhesion molecules, such as integrins.[5–8] Vascular endothelial growth factor is considered an important vasculogenic mediator of embryonic and postnatal angiogenesis that functions in the promotion of endothelial cell growth and/or inhibition of apoptosis.[9] Elevated VEGF concentrations have been reported in several types of metastatic cancers, suggesting that it plays a role in cancer progression.[10] FGF-2 has been reported to be a potent stimulator of angiogenesis *in vitro* and is found in the serum and/or urine of patients with several types of cancer, including leukemias.[11,12] Angiogenesis has recently been reported in hematological malignancies, such as leukemia and myelodysplastic syndromes.[11,13–18] Vacca *et al.* reported finding positive correlations between increased bone marrow microvessel density (MVD) and both the plasma-cell labeling index and disease activity in MM patients.[19] The increased plasma or serum concentrations of FGF-2 and VEGF has been observed not only in solid tumors, such as prostate

cancer and renal cancers, but also in hematological malignancies, such as acute lymphocytic leukemia, acute myeloleukemia, chronic myeloleukemia, and also MM.[11 13 18] Thus, it is critical to determine whether antiangiogenetic therapy will be useful for the treatment of hematological malignancies.

Thalidomide was introduced in the 1950s as a sedative, but was withdrawn from the market in the 1960s because of its teratogenicity.[20] Recently, thalidomide has been found to be effective against erythema nodosum leprosum,[21] graft-versus-host disease[22] and Crohn's disease.[23] An antiangiogenic function of thalidomide in a rabbit cornea micropocket assay was reported by D'Amato *et al.* in 1994.[24] Rajkumar *et al.* reported that tumor angiogenesis occurs in the bone marrow of MM patients and that thalidomide therapy improved the MM in some previously treated myeloma patients.[25]

We recently reported that the elevated level of plasma FGF-2 in MM patients correlated with increased disease activity.[26] Furthermore, we showed that thalidomide was effective for the treatment of refractory MM in Japan.[27] Hence, in the present study, we assessed the MVD of the bone marrow of untreated MM patients to confirm tumor angiogenesis in MM. We also investigated the effectiveness of thalidomide treatment for refractory MM patients and whether thalidomide administration altered tumor angiogenesis.

MATERIALS AND METHODS

Patients

To study angiogenesis in MM, we evaluated 51 bone marrow biopsy specimens from 51 untreated MM patients at Keio University Hospital. The clinical characteristics of the patients are shown in Table 1. The 51 patients consisted of 28 males and 23 females, and their median age was 62 years. Forty-two cases fulfilled the diagnostic criteria for MM of the South West Oncology Group (SWOG), and the other nine cases were diagnosed with a monoclonal gammopathy of undetermined significance (MGUS) based on the SWOG criteria.[28] The major subclass of immunoglobulin heavy chains was IgG (32 out of 51 cases). We used six bone marrow specimens, two from transplantation donors and four obtained at autopsy from patients with non-hematological diseases (pneumonia in two cases and cancer without bone marrow involvement in the other two cases).

To evaluate the effect of thalidomide on angiogenesis, we analyzed bone marrow biopsy specimens before and after thalidomide administration to 11 patients with refractory MM (six females and five males) who had been treated with conventional chemotherapy, such as vincristine, melpharan, prednisolone and dexamethasone (Table 2). Two patients also received peripheral blood stem cell transplantation and Interferon alpha (IFNα) before being treated with thalidomide. The disease of all 11 patients was categorized as Durie-Salmon stage 3 before treatment with thalidomide.[29]

Table 1 Clinical parameters of untreated patients

Number of patients	51
Average age (years)	62 (range, 37–88)
Male/Female	28/23
Subclass of Ig	
IgG	32
IgA	11
IgD	2
BJP	5
Nonsecretory	1
Stage (Durie-Salmon)	
1	15
2	6
3	21
MGUS	9

BJP, Bence Jones proteins; Ig, immunoglobulin; MGUS, monoclonal gammopathies of undetermined significance.

Immunohistochemistry

Sections of paraffin-embedded bone marrow specimens were stained by a standard indirect immunohistochemical technique with anti-CD34 mouse monoclonal antibody (Novocastra, Newcastle, UK) to highlight endothelial cells. The myeloma cells in the bone marrow were stained with antihuman plasma cell mouse monoclonal antibody (VS38c; DAKO, Glostrup, Denmark). The bone marrow specimens of the patients treated with thalidomide were also stained with anti-FGF-2 rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-FGF Receptor 1 rabbit polyclonal antibody generated by immunizing a rabbit with a synthetic oligopeptide of human FGF Receptor 1.[30]

Measurement of bone marrow microvessel density and the grading of myeloma cell invasion

All bone marrow biopsy specimens were evaluated for cellularity by light microscopy with a 10× power objective lens. Five areas with high cellularity were randomly selected and examined with an 80× power objective lens. Five fields were taken, with each field representing an area of 0.108 mm^2. Individual microvessels (stained brown by immunohistochemistry anti-CD34 antibody) were counted in each fields and their density was calculated.

The grade of myeloma cell invasion in the bone marrow was determined by both hematoxylin–eosin (HE) staining and immunohistochemistry with anti-VS38c antibody. Invasion was graded as 'mild' if myeloma cells accounted for less

Table 2 Summary of patients treated with thalidomide

Patient	Age (years)	Sex	Immunoglobulin type	Stage	Previous treatment
1	46	M	IgG kappa	3A	MP, VAD, ABMT, dexamethasone, MCNU-VMP
2	45	M	IgA kappa	3A	VAD, EDAP, PBSCT, IFNα, radiation
3	55	F	IgG kappa	3A	VAD, PBSCT, dexamethasone, IFNα
4	57	F	BJP lamda	3B	L-PAM + ADR + dexamethasone, VAD
5	58	M	IgG kappa	3A	VAD, VCAP
6	58	M	IgA lamda	3A	VAD, dexamethasone, MP, VP-16
7	70	F	IgG kappa	3A	Dexamethasone, VCAP
8	70	F	IgG kappa	3A	Dexamethasone, melphalan, VCAP
9	63	F	IgG kappa	3A	MP, VAD
10	59	M	IgA kappa	3A	VAD, dexamethasone, VCAP
11	55	F	IgA lamda	3A	Dexamethasone, VCAP, VCAD

AMBT, autologous blood and marrow transplantation; EDAP, etoposide, cisplatinum, dexamethasone and ara-C; F, female; Ig, immunoglobulin; L-PAM + ADR, melphalan and adriamycin; M, male; MCNU, ranimustine; MP, melphalan and prednisolone; PBSCT, peripheral blood stem cell transplant; VAD, vincristine, doxorubicin and dexamethasone; VCAD, vincristine, cyclophosphamide, adriamycin and dexamethasone; VCAP, vincristine, cyclophosphamide, doxorubicin and prednisolone; VMP, vincristine, melphalan and prednisolone.

than 25% of all nucleated bone marrow cells, 'severe' if the myeloma cells accounted for over 75% of all nucleated bone marrow cells, and 'moderate' if the invasion was between 'mild' and 'severe'.

Estimation of VEGF and FGF-2 concentrations in MM patients' plasma

Plasma samples were collected from 10 patients with refractory myeloma (cases 2–11) before and after 2–4 weeks of thalidomide administration. FGF-2 and VEGF were measured with an enzyme-linked immunosorbent assay (ELISA) system (R&D Systems, Minneapolis, MN, USA). Briefly, the plasma was collected and, after adding EDTA as an anticoagulant, was stored at −80°C. Patients' samples were applied to microtiterplates coated with a specific monoclonal antibody, and they were incubated at room temperature for 2 h. The plates were then washed three times and, after adding peroxidase-conjugated secondary polyclonal antibodies specific for the primary antibodies to the wells, they were incubated at room temperature for 2 h. After washing the wells, a substrate solution was added and the intensity of the blue color products was measured at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). The limit of detection of FGF-2 and VEGF in plasma was 1 pg/mL and 15.6 pg/mL, respectively. The FGF-2 and VEGF concentrations were considered elevated if they exceeded the highest value in the healthy control group. The cutoff values of FGF-2 and VEGF were 7.67 pg/mL and 38.3 pg/mL, respectively.

Thalidomide treatment and assessment of therapeutic effectiveness

Thalidomide was supplied by Sociedade Farmaceutic Brasifa Ltda. (Rio de Janeiro, Brazil) and administered *per os* at a dose of 200 mg/day for 7 days. No chemotherapeutic agents, including steroids, or radiotherapy was administered during thalidomide treatment. Certain supportive therapies, including blood transfusion, granulocyte colony-stimulating factor (G-CSF), supplemental gamma globulin, and/or pamidronate disodium administration were permitted concomitantly. If no serious side-effects were observed during the first week, the dose of thalidomide was increased to 400 mg and continued as a maintenance dose. When side-effects, such as granulocytopenia, were observed in patients after administering the increased dose of thalidomide, the dose was decreased to 200 mg/day. The effectiveness of thalidomide was evaluated by classifying the patients according to their response into the following groups: a responsive group, with a more than 30% decrease in serum M-protein or daily urine Bence Jones protein concentration sustained for at least 4 weeks; a stable group, with a less than 25% change in M-protein level; and a progressive group, with a more than 30% increase in M-protein level.

RESULTS

Microvessel density of the bone marrow of untreated patients

The MVD of the bone marrow of the 51 patients with MM or MGUS and the six control patients with non-hematological disorders is shown in Fig. 1. The typical histological findings of the bone marrow observed while counting microvessels are shown in Fig. 2. The MVD of the bone marrow in the control group was 20.4–70.9 vessels/mm^2 ($n = 6$, 43.5 ± 20.3 vessels/mm^2). The MVD of the bone marrow of MGUS patients varied widely, from 38.9 to 133.3 vessels/mm^2 ($n = 9$, 78.2 ± 30.1 vessels/mm^2), and the range in the MM patients was 38.9–264.8 vessels/mm^2 ($n = 42$, 111.1 ± 60.1 vessels/mm^2). Thus, the MVD of the BM of the MM patients was

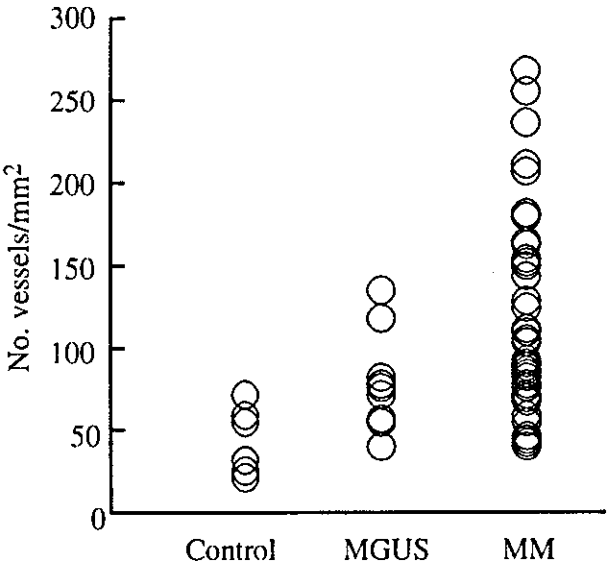

Figure 1 Microvessel density (MVD) of the bone marrow of untreated multiple myeloma (MM) patients. The MVD of the bone marrow of 51 patients and six non-hematological control patients are shown. The MVD was calculated as the number of CD34-positive microvessels observed in five fields (area of each field: 1.08 mm^2) in the bone marrow under the 80x lens of a light microscope. The MVD was 20.4–70.9 vessels/mm^2 in the control group ($n = 6$), 38.9–133.3 vessels/mm^2 in the monoclonal gammopathy of undetermined significance (MGUS) patients ($n = 9$), and 38.9–264.8 vessels/mm^2 in the MM patients ($n = 42$).

higher than in the controls; however, the increases in MVD in the MM and MGUS patients' bone marrow were not statistically significant compared with the controls.

Representative histological findings in the bone marrow of an untreated MM patient and normal bone marrow are shown in Fig. 2. Myeloma cell invasion in the bone marrow was graded by examination of HE-stained sections (Fig. 2a–d) and sections immunohistochemically stained with antiplasma cell antibody VS38c (Fig. 2e–h). There were no differences between the numbers of CD34-positive microvessels in the bone marrow with 'mild' invasion by myeloma cells and in normal bone marrow; however, there were clear increases in the number of microvessels in the bone marrow with a 'moderate' or 'severe' invasion by myeloma cells (Fig. 1). The marrow space of the bone marrow with 'severe' invasion had been replaced by numerous infiltrating myeloma cells accompanied by fibrosis. There was a marked increase in the number of microvessels in the bone marrow with a 'severe' invasion compared with normal bone marrow.

To evaluate the tendency toward increased angiogenesis in the bone marrow of MM patients statistically, we examined the MVD values of bone marrow with mild, moderate, and severe invasion by myeloma cells, and normal bone marrow. The results showed that MVD increased with the grade of myeloma cell invasion (Fig. 3). The mean MVD of normal bone marrow and the mild invasion cases was 43.5 and 79.5 vessels/mm^2, respectively, as opposed to 113.1 and 167.4 vessels/mm^2 in the moderate and severe invasion cases, respectively. The MVD of bone marrow in the moderate invasion cases and the severe invasion cases was significantly higher than in normal bone marrow ($P < 0.05$ and $P < 0.005$, respectively).

Thalidomide treatment and angiogenesis in bone marrow

Eleven refractory cases (six females and five males) that had been treated with traditional chemotherapy were treated with thalidomide (Table 2). Figure 4a shows the concentrations of M-protein in serum (cases 1–3, 5–11) and urine (case 4) before and after thalidomide treatment. Cases in which the M-protein concentration in serum or urine decreased to below 70% after thalidomide treatment were defined as 'responsive' cases. Cases with over 130% of the initial M-protein concentration after treatment were defined as 'progressive' cases. Cases with 70–130% of the initial concentration of M-protein after treatment were defined as 'stable' cases. Of the 11 cases treated with thalidomide, seven (64%) were 'responsive', three were 'stable', and one was 'progressive'. The urine concentration of M-protein in case 4 was 6010 mg/dL before treatment with thalidomide, and decreased to 1050 mg/dL after 4 weeks of treatment. In case 1, the concentration of serum M-protein changed from 5940 mg/dL to 2394 mg/dL after administration of thalidomide. In the 'responsive' cases, the grade of plasma cell invasion in the bone marrow was lower or unchanged after treatment. In the 'stable' and 'progressive' group, the grade of plasma cell invasion was unchanged or had increased after thalidomide treatment. There was an obvious decrease in the degree of bone marrow invasion in some of the patients in whom thalidomide treatment was effective.

Figure 4b shows the MVD of the bone marrow before and after thalidomide therapy. In the seven 'responsive' cases, MVD decreased in three cases (cases 6, 9 and 11) and increased in three cases (cases 1, 4, and 5) and was stable in one case (case 10). The MVD of case 10 was lower than the other cases before and after the treatment with thalidomide. The MVD increased in all of the 'stable' cases (cases 3, 7 and 8), but decreased in the 'progressive' case (case 2). No tendency toward a correlation between the effectiveness of thalidomide and the changes in the MVD of the bone marrow after thalidomide treatment was found.

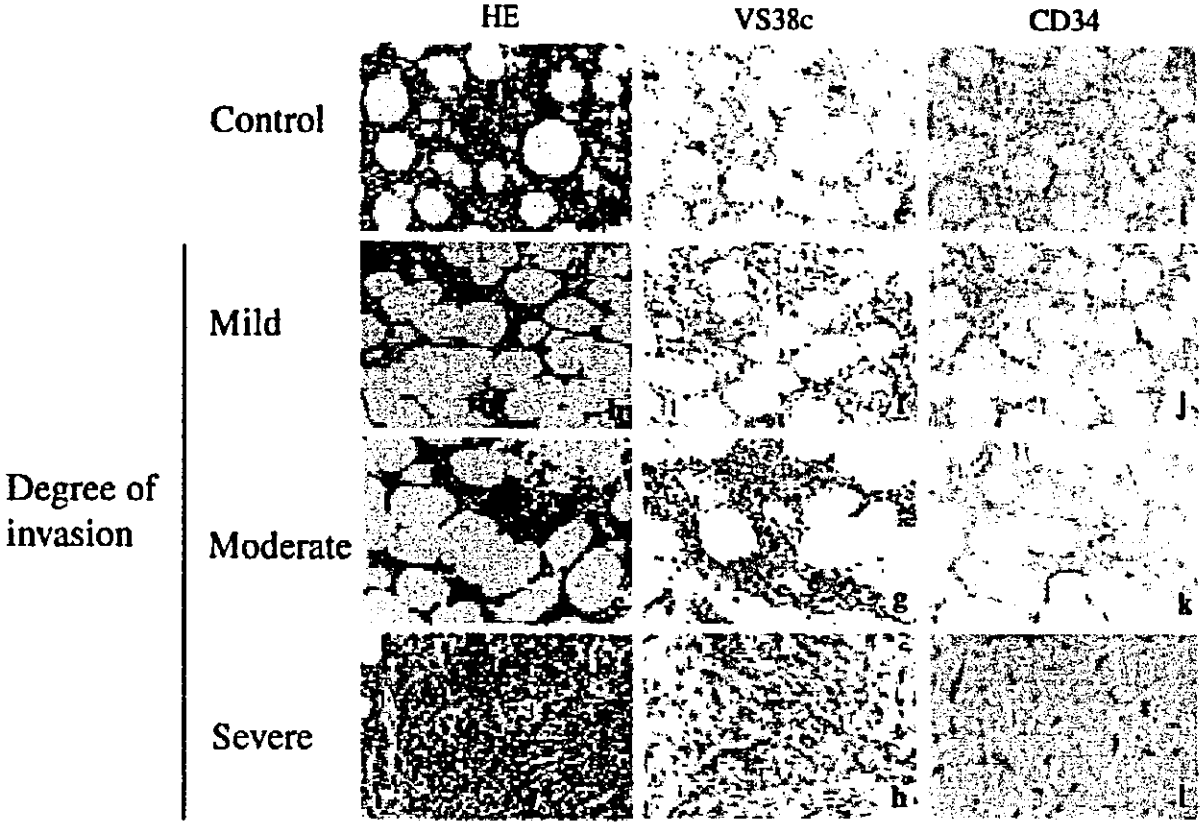

Figure 2 Grading of myeloma cell invasion and angiogenesis in the bone marrow of untreated multiple myeloma (MM) patients. Representative histologies of the bone marrow of an untreated MM patient and normal bone marrow are shown. Myeloma cell invasion in the bone marrow was graded by examining (**a,b,c,d**) sections stained with hematoxylin–eosin (HE) and (**e,f,g,h**) sections immunohistochemically stained with antiplasma cell antibody, VS38c. (**i,j,k,l**) Immunohistochemical staining with anti-CD34 antibody was performed to visualize the blood vessels. The number of microvessels in the bone marrow with 'moderate' or 'severe' myeloma cell invasion was obviously increased (**k,l**). In the bone marrow with 'severe' invasion, the marrow space was replaced by numerous infiltrating myeloma cells and accompanied by a fibrosis. There was a marked increase in the number of microvessels in the bone marrow with 'severe' invasion, compared with normal marrow (**l**).

Plasma FGF-2 and VEGF concentrations after thalidomide treatment

The plasma concentrations of angiogenic factors, FGF-2 (bFGF) and VEGF, were measured by ELISA in 10 patients treated with thalidomide. Before thalidomide treatment, the plasma FGF-2 concentrations in all of the cases in which FGF-2 was detectable (nine cases) exceeded the normal range (FGF-2 was not detectable in the plasma in case 4) (Fig. 5a). The highest plasma value was 278 pg/dL, in case 6. After 2–4 weeks of thalidomide treatment, the FGF-2 concentration had decreased in all nine cases, including the progressive case (case 2) with an increased serum M-protein concentration and the cases with increased MVD of the bone marrow (cases 3,5,7 and 8) after treatment with thalidomide.

Plasma VEGF concentrations were also determined by ELISA (Fig. 5b). Before the thalidomide therapy, an increased VEGF concentration of plasma was seen in nine cases, and the highest value was 208 pg/dL (case 5). In these nine cases, after 2–4 weeks of thalidomide treatment, a decrease of VEGF concentration was observed in eight cases, and the plasma VEGF concentration in six of these eight cases was within the normal range. These decreases in plasma FGF-2 or VEGF concentration may also have been caused by thalidomide.

Representative histological sections of bone marrow before and after thalidomide treatment in case 9, a 'responsive' case, are shown in Fig. 6. The myeloma cell invasion in the bone marrow had improved after treatment, and the number of CD34-positive microvessels had decreased. Immunohistochemistry with anti-FGF-2 antibody revealed that the cytoplasm of many hematopoietic cells, including myeloma cells, was positive (Fig. 6). There were no significant changes in the FGF-2 staining pattern after thalidomide treatment, despite the decreased concentration of FGF-2 after treat-

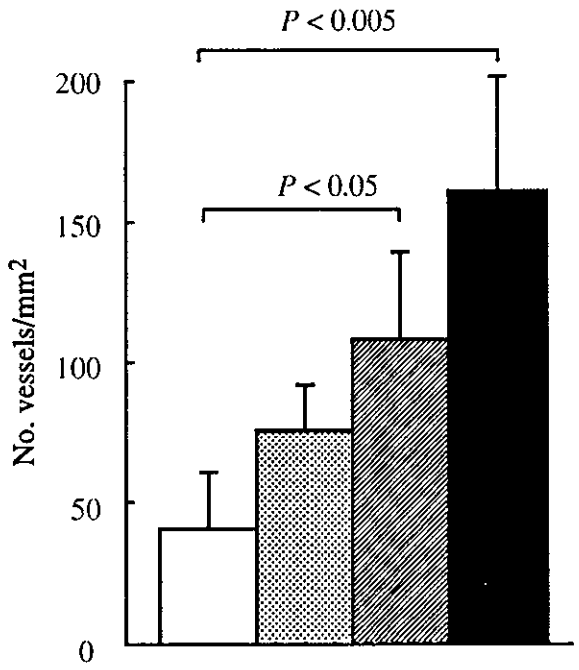

Figure 3 Increased microvessel density (MVD) in the bone marrow of multiple myeloma (MM) patients. The MVD was measured in the bone marrow with mild, moderate and severe invasion of myeloma cells and in the normal bone marrow in order to statistically evaluate the tendency toward increased angiogenesis in the bone marrow of MM patients. The MVD increased with the grade of myeloma cell invasion. The mean MVD in normal bone marrow and mild invasion cases was 43.5 and 79.5 vessels/mm^2, respectively, as opposed to 113.1 and 167.4 vessels/mm^2 in the moderate and severe invasion cases, respectively. The increased MVD in the moderate and severe invasion cases were significant compared with normal bone marrow ($P < 0.05$ and $P < 0.005$, respectively). (□), Control ($n = 6$); (▨), mild invasion ($n = 14$); (▨), moderate invasion ($n = 28$); and (■) severe invasion ($n = 9$).

ment. In contrast, FGF Receptor 1 was also widely expressed by hematopoietic cells, including myeloma cells (Fig. 6). No alterations in FGF Receptor 1 expression were seen after treatment.

DISCUSSION

The angiogenesis in solid tumors has been thought to play a role in tumor growth, invasion and metastasis.[31] In hematological malignancies, the tumor cells invade the bone marrow space and proliferate, and they replace the normal hematopoietic area because the bone marrow space is limited by the surrounding trabeculae of bone. The results of the present study showed that the MVD of the bone marrow of MM patients was higher than in the healthy controls, and greater angiogenesis was observed in MM patients with higher grades of myeloma cell invasion of the bone marrow. These findings suggest that the relationship between angiogenesis and development of MM is similar to their relationship in solid tumors.[31] Although it is not yet clear whether angiogenesis is indispensable to the pathogenesis of the disease, if angiogenesis is necessary for the development of MM, inhibition of angiogenesis may be a useful means of treatment.[19]

Vacca *et al.* reported a positive correlation between angiogenesis and the disease activity of MM.[32] Furthermore, the increased angiogenesis in bone marrow and increased levels of stimulators of angiogenesis, including FGF-2, VEGF and HGF, have recently been reported in human leukemia patients.[6,33] This has led to discussion of the possibility of antiangiogenic therapy for hematological malignancies. Thalidomide has been used to treat some MM patients as a new therapy for MM because it has an antiangiogenic effect. By using a rabbit cornea micropocket assay, D'Amato *et al.* has shown that thalidomide inhibits FGF-2-induced angiogenesis.[24] Thalidomide has also been reported to suppress production of tumor necrosis factor alpha by macrophages and to stimulate production of interleukin-2, -4, -10, and IFNγ.[34–37] These immunomodulating functions of thalidomide may contribute to the suppression of the survival and/or growth of myeloma cells. Tosi *et al.* have reported that thalidomide may suppress the progression of MM via impaired production of VEGF by myeloma cells.[38]

In the present study, we have shown that thalidomide may be effective for impairing tumor angiogenesis in the bone marrow of MM patients whose disease is refractory to conventional chemotherapy and that thalidomide reduced the plasma FGF-2 and VEGF level in almost all refractory MM cases. The cause of the decreased level of FGF-2 after thalidomide administration is unknown.

In the present study, we considered the relationship between plasma angiogenic factor (FGF-2, VEGF) level and MVD in the bone marrow of the refractory MM cases treated with thalidomide. Increased MVD was observed in the bone marrow after administration of thalidomide in some patients despite the depressed disease activity and the decreased concentrations of FGF-2 and VEGF. This discrepancy may be caused by the assessment procedure for tumor angiogenesis in the bone marrow. Singhal *et al.* also could not demonstrate a clear relationship between the bone marrow MVD and the response to the treatment with thalidomide in myeloma.[39] Hlatky *et al.* has stated that the efficacy of antiangiogenic agents cannot be simply visualized by alterations in MVD during treatment because the MVD may be outward and influenced by shrinkage, necrosis or apoptosis of the tumor.[40]

Immunohistochemistry showed that many of the hematopoietic cells and myeloma cells produced the FGF-2 protein. The FGF-2 receptors are also expressed in hematopoietic cells and myeloma cells. These results may show an auto-

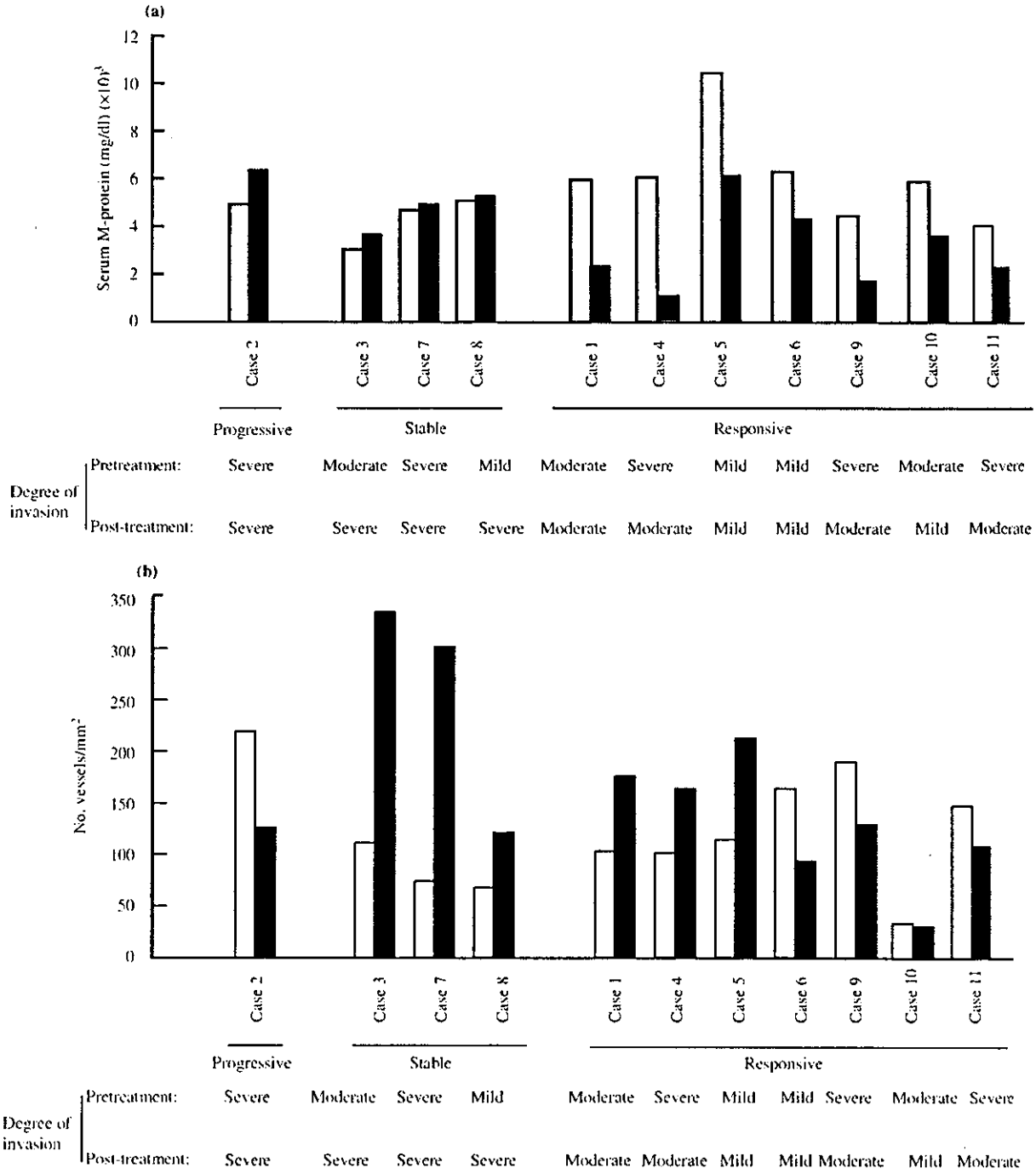

Figure 4 Effect of thalidomide treatment and change of microvessel density (MVD) of the bone marrow. (a) Changes in serum M-protein after thalidomide treatment. The changes in concentration of M-protein in serum (cases 1–3, 5–11) and urine (case 4) after thalidomide administration are shown. The cases were classified into three groups according to the effect of thalidomide: 'responsive'; 'stable'; and 'progressive', based on the degree of change in M-protein concentration. There were seven 'responsive' cases, three 'stable' cases, and one 'progressive' case. (b) Changes in MVD of the bone marrow after thalidomide treatment. The MVD of the bone marrow before and after thalidomide treatment are shown. In the 'responsive' group, MVD decreased in four cases (cases 6, 9, 10 and 11) and increased in three cases (cases 1, 4 and 5). The MVD increased in all of the 'stable' cases (cases 3, 7 and 8), but decreased in the 'progressive' case (case 2) despite the increased disease activity. (▯), Pretreatment of thalidomide; (■), post-treatment of thalidomide.

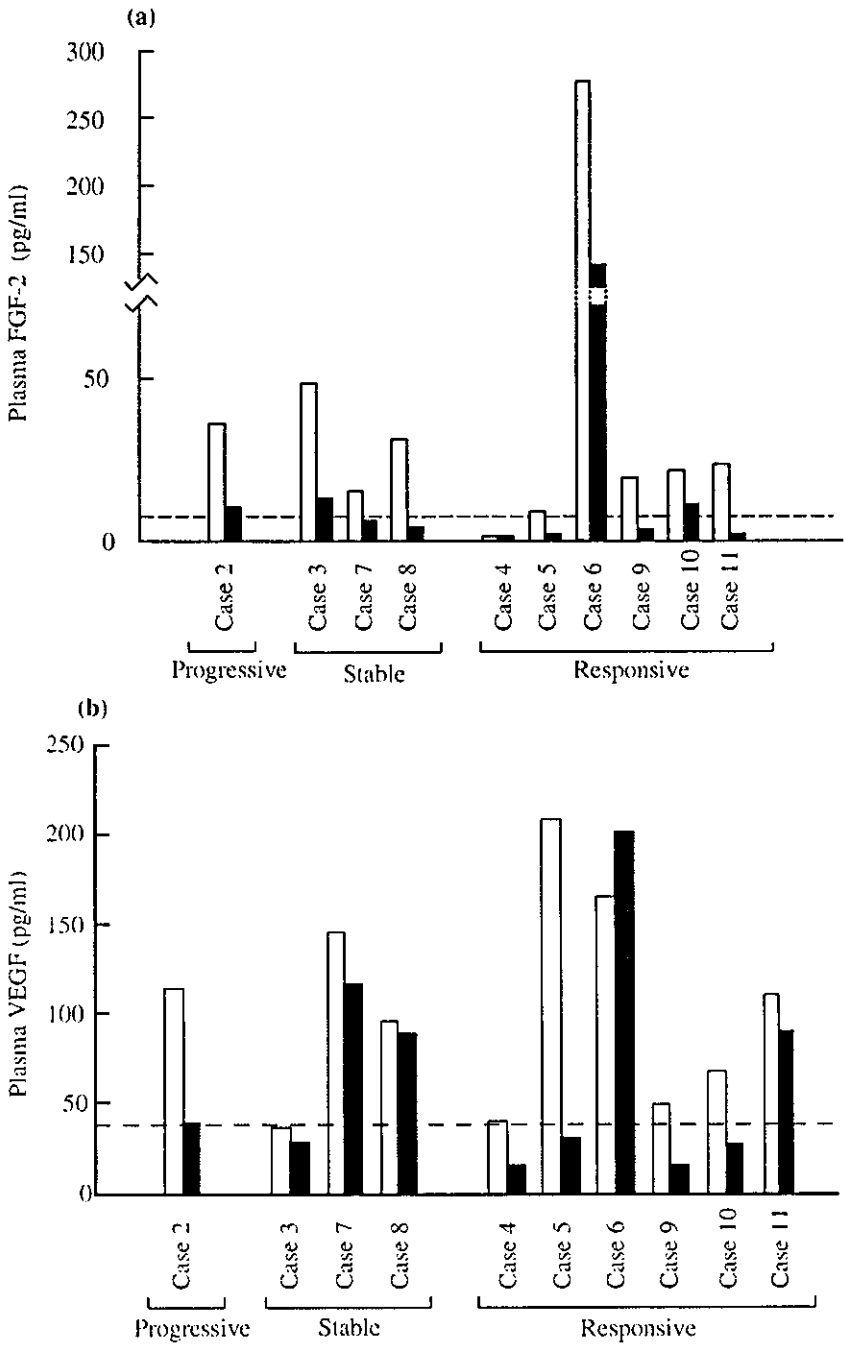

Figure 5 Plasma fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) concentrations of multiple myeloma (MM) patients before and after thalidomide treatment. The concentrations of FGF-2 and VEGF were determined in 10 of the 11 cases by enzyme-linked immunosorbent assay. (a) Plasma FGF-2 concentrations. Before thalidomide treatment, the FGF-2 concentration in all cases in which FGF-2 was detectable (9 cases) was higher than in the healthy subjects. The highest value was 278 pg/dL, in case 8. The FGF-2 concentration decreased in all cases after thalidomide treatment, and in five cases (cases 5, 7, 8, 9 and 11) it decreased to below the upper limit in the healthy subjects. FGF-2 was not detectable in case 4. (b) Plasma VEGF concentration. Before thalidomide administration, the VEGF concentration in eight cases (cases 2, 5, 6, 7, 8, 9, 10 and 11) was higher than in the healthy subjects. The highest value was 208 pg/dL, in case 5. After thalidomide treatment, a decrease in VEGF concentration was observed in eight cases, and in five cases it decreased to below the upper limit in the healthy subjects. (---), Average of plasma cytokine; (□), pretreatment of thalidomide; (■), post-treatment of thalidomide.

crine loop in which the FGF-2 produced by myeloma cells affects the growth or survival of the myeloma cell. The high concentration of FGF-2 in the patients' plasma and the possible existence of an FGF-2 autocrine loop in myeloma cells suggests the possibility of using anti-FGF-2 and/or anti-FGF-2 receptor antibody as a new form of therapy.[41] The bone marrow specimens of the MM patients treated with thalidomide also stained with anti-VEGF antibody. However, there was no positive finding in all specimens because of the manipulation of decalcification.

In the present study, we showed that seven out of 11 refractory MM cases were responsive, three cases were stable, and one case was progressive for the treatment of thalidomide. In the three stable cases, two patients were of advanced age (cases 7 and 8). The patient in the progressive case (case 2) was previously treated with irradiation and

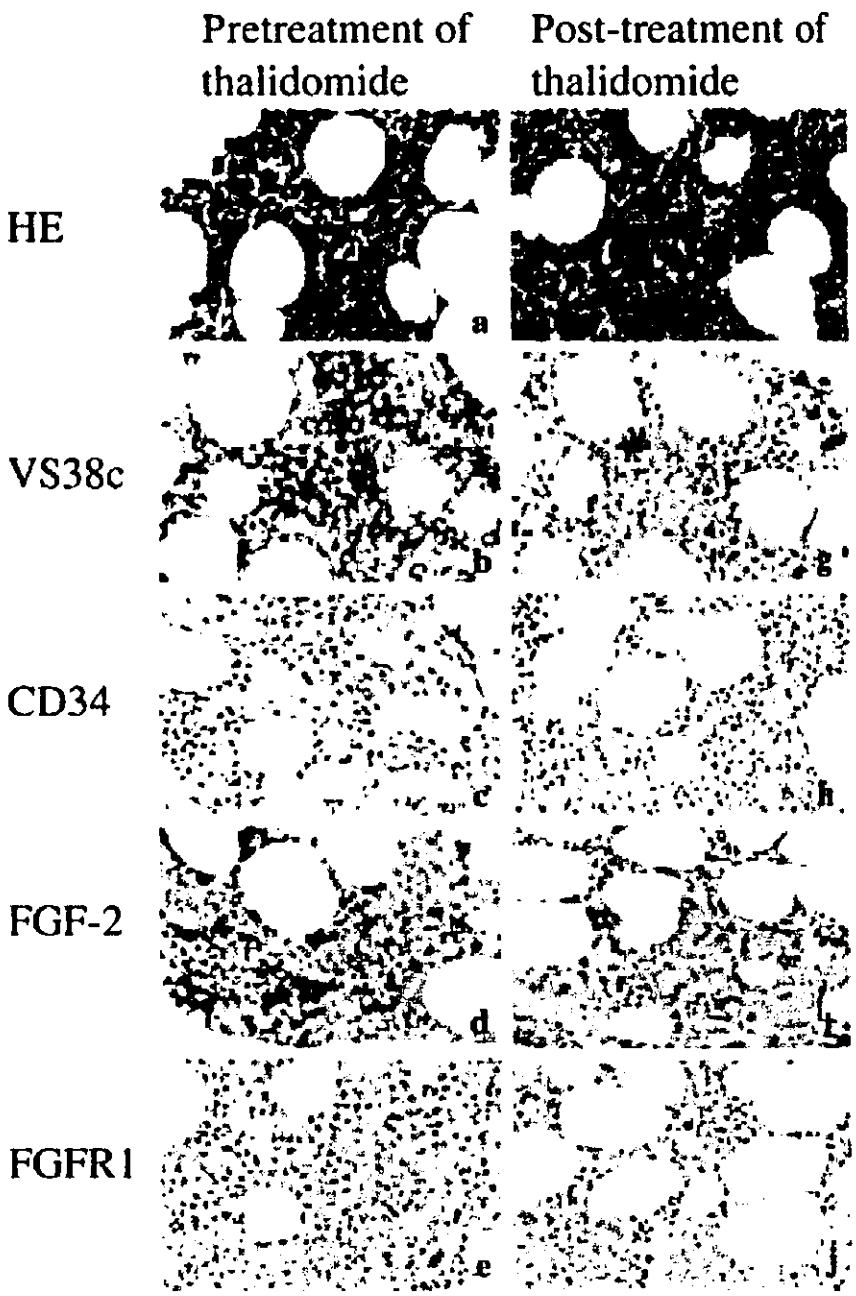

Figure 6 Histologies of the bone marrow before and after thalidomide treatment. Representative histologies of the bone marrow in case 9, a 'responsive' case, before and after thalidomide treatment are shown. (**a**,**b**,**f**,**g**) The myeloma cell invasion of the bone marrow improved after treatment. (**c**,**h**) The number of CD34-positive microvessels also decreased after treatment. Expression of fibroblast growth factor (FGF-2) was observed in the cytoplasm of hematopoietic cells and myeloma cells. (**d**,**i**) There was no significant change in the FGF-2 staining pattern after thalidomide treatment. (**e**,**j**) FGF Receptor 1 was also widely expressed in hematopoietic cells and myeloma cells, but there were no changes in its expression after treatment.

showed severe anemia before the treatment of thalidomide. Therefore, the response to the thalidomide may be influenced by age and previous therapies.

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ORIGINAL PAPER

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Primary central nervous system lymphoma in Japan 1995–1999: changes from the preceding 10 years

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Abstract *Purpose*: Previously, we conducted a nationwide survey of primary central nervous system lymphoma (PCNSL) treated between 1985 and 1994 in Japan. In the present study, we conducted further investigations of PCNSL patients treated between 1995 and 1999 to clarify possible changes with time in the clinical features, treatment, and outcome of this disease. *Methods*: Thirteen Japanese institutions were surveyed, and data on 101 patients with histologically-confirmed PCNSL were collected. These data were compared with those of 167 patients treated at the same institutions between 1985 and 1994. *Results*: Regarding patient and tumor characteristics, the proportion of patients with good performance status (PS) was significantly higher in the group treated during 1995–1999 than in that treated during 1985–1994, but other characteristics were not significantly different. Regarding treatment, more patients in the more recent period (66%) received systemic chemotherapy than those in the preceding period (53%, $P = 0.049$). For all patients, including those who did not complete radiotherapy, the median survival time was 17 months and 30 months in patients treated between 1985 and 1994 and those treated between 1995 and 1999, respectively, and the 5-year survival rate was 15% versus 31% ($P = 0.0003$). In both patient groups, higher age and tumor multiplicity were associated with poor prognosis in multivariate analysis. In patients treated between 1995 and 1999, those who received systemic chemotherapy showed significantly better prognosis than those who did not ($P = 0.0049$), but the difference was not significant in multivariate analysis ($P = 0.23$). *Conclusions*: The high survival rates observed in the present survey are comparable with those of recent prospective studies employing intensive chemoradiotherapy. The improvement in prognosis appeared to result, at least in part, from the increase in the proportion of patients with better PS. Since the clinical feature and treatment outcome of patients with PCNSL can thus change with the era, historical control data should not be used in comparing different treatment modalities.

Keywords Brain neoplasm · Lymphoma · Primary CNS lymphoma · Radiotherapy · Chemotherapy

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Introduction

Primary central nervous system lymphoma (PCNSL) is increasing and is becoming one of the most important tumors in neuro-oncology. Radiation therapy has been the standard treatment for PCNSL until recently, but the outcome of patients treated by radiation alone has not necessarily been satisfactory (Shibamoto et al. 1990; Reni et al. 1997; Hayabuchi et al. 1998; Nelson 1999). More recently, the use of high-dose methotrexate (MTX)-containing chemotherapy before radiation appeared to have gained some success in obtaining

long-term survival (Glass et al. 1994; Blay et al. 1998; Brada et al. 1998; Abrey et al. 2000; Ferreri et al. 2000; O'Brien et al. 2000; Reni et al. 2001; Bessel et al. 2001; Caldoni & Aebi 2002; DeAngelis et al. 2002). However, there has been no randomized trial suggesting the superiority of the combined modality treatment over radiation therapy alone, and a recent study by a German group suggested a high rate of progressive disease during treatment with 6 courses of 8 g/m^2 of MTX (Herrlinger et al. 2002). Therefore, the benefit of high-dose MTX appears to remain uncertain. Since the clinical features of PCNSL appear to be changing with time, it may not be reasonable to consider that combined MTX-containing chemotherapy and radiation is superior to radiation alone, by comparing the results of combined treatment with the historical control data in patients treated by radiation therapy alone.

Previously, Hayabuchi et al. (Hayabuchi et al. 1998) conducted a nationwide survey of PCNSL in Japan treated between 1985 and 1994. The findings on 466 patients were previously published. Considering the increasing importance of this disease, we organized a research group consisting of 13 institutions to carry out both retrospective and prospective studies on PCNSL. As a first study of this group, we collected data on PCNSL patients treated between 1995 and 1999 at these institutions. In addition to analyzing these data on 101 patients, we compared the data with those on 167 patients from the previous survey treated between 1985 and 1994 at the same institutions, to investigate changes in the clinical feature, treatment modality, and outcome between these eras.

Materials and methods

Subjects of the present survey were patients with histologically-proven PCNSL who received radiation therapy between 1995 and 1999. Those who did not complete the planned radiotherapy were included. Clinical characteristics, treatment and prognosis of each patient shown in the Results section were asked using a detailed questionnaire. Data on 101 patients were collected from 13 institutions. For comparison, data on 167 patients treated in the preceding 10 years, i.e., between 1985 and 1994, at the same institutions were obtained from the data source of the previous nationwide survey (Hayabuchi et al. 1998) and were analyzed. Data regarding tumor size (maximum diameter at diagnosis and before radiation therapy) was asked for in the present survey, which had not been done in the previous survey. As often happens with such a survey, a number of the items were unanswered by the investigators. Various chemotherapy regimens had been used, and were categorized as follows: (A) cyclophosphamide, vincristine, and prednisolone (COP) or COP plus doxorubicin (CHOP/VEPA); (B) intravenous methotrexate (MTX) alone or MTX-containing regimens. The drugs included in regimen A had often been used in combination with MTX, and such regimens were categorized into this group; (C) cytarabine plus procarbazine; (D) nitrosourea-containing regimens. Some of the drugs in regimen A had been used in combination with nitrosoureas, and such regimens were included in this group. When MTX had been used in combination, the regimen was categorized into group B; (E) cisplatin plus etoposide; and (F) Single use or combination of miscellaneous other agents not included in the above groups. For analysis of treatment results, regimens C–F were grouped together. Differences in patient, tumor, and treatment characteristics between groups were examined by Fisher's exact test.

Survival rates were calculated from the date of starting radiotherapy using the Kaplan-Meier method, and differences in pairs of survival curves were examined by the log-rank test. Multivariate analysis of prognostic factors was carried out using the Cox proportional hazards model. In doing multivariate analysis, patients were divided into two groups, and all the parameters were entered as dichotomous variables. All statistical analyses were carried out using a computer program, Stat View Version 5 (SAS institute, Cary, NC, USA).

Results

Table 1 shows patient, tumor, and treatment characteristics in the two groups treated between 1985 and 1994 and between 1995 and 1999. There were more patients with better WHO performance status (PS) score in the group treated between 1995 and 1999 than in the

Table 1 Patient, tumor, and treatment characteristics

Characteristic		1985–1994	1995–1999	*P*
Gender	Male/female	97/70	67/34	0.20
Age (years)	< 60/≥ 60	83/84	53/48	0.71
	Median (range)	60 (15–84)	59(15–84)	
Performance status	0–2/3,4	69/95	60/41	0.0078
Lactate dehydrogenase	Normal/high	49/34	50/30	0.75
B symptom	Yes/no	16/133	11/81	0.83
Phenotype	B/T	75/8	79/6	0.59
Tumor number	Single/multiple	103/63	56/43	0.44
Maximum tumor diameter	At diagnosis	–	3 (1.5–9)	
Median (range) (cm)	Before radiation	–	3 (0–9)	
Radiotherapy	Completed/not completed	158/9	97/4	0.77
Radiation field	Whole brain/partial brain	146/21	92/9	0.43
Spinal radiation	Yes/no	15/152	4/97	0.15
Total dose (Gy)	< 50/≥50	54/113	28/73	0.49
	Median (range)	50 (2–70)	50 (6–80)	
Whole-brain dose (Gy)	< 40/≥40	70/97	42/59	1.0
	Median (range)	40 (0–54)	40 (0–60)	
Chemotherapy	Yes / no	78/70	65/34	0.049

Table 2 Chemotherapy regimens (COP cyclophosphamide, vincristine and prednisone, CHOP/VEPA COP plus doxorubicin)

Regimen	1985–1994	1995–1999
COP, CHOP/VEPA	35 (45%)	25 (38%)
Methotrexate-containing regimens	18 (23%)	27 (42%)
Cytarabine and procarbazine	0	7 (11%)
Nitrosourea-containing regimens	13 (17%)	2 (3%)
Cisplatin and etoposide	8 (10%)	4 (6%)
Miscellaneous drugs	4 (5%)	0

group treated in the preceding 10 years, but the other patient and tumor characteristics did not differ significantly between the two groups. Radiotherapy characteristics were similar between the two groups. During both study periods, more than 85% of the patients were treated with whole-brain irradiation with or without focal boost, and the median total and whole brain doses were 50 Gy and 40 Gy, respectively. Whole spinal irradiation was employed in less than 10% of the patients. On the other hand, more patients seen between 1995 and 1999 received systemic chemotherapy than those seen between 1985 and 1994 (66% vs 53%, $P = 0.049$). Table 2 shows chemotherapy regimens used in the two groups. The use of MTX-containing regimens appeared to be increasing recently. However, a high dose of MTX (> 2 g/m^2 per administration) was used in only 14 patients (14% of all patients) treated between 1995 and 1999.

Figure 1 shows overall survival curves for all patients in the two groups. Patients in the present survey had significantly better survival rates than those in the previous survey ($P = 0.0003$); median survival time was 30 vs 17 months, and the 3-year survival rate was 46% vs 24%. The 5-year survival was 31% and 15%, respectively. Table 3 summarizes survival data in the two groups according to potential prognostic factors. In both study periods, patients with ages < 60 years, PS 0–2, or a single tumor showed significantly higher survival rates. Patients with normal lactate dehydrogenase (LDH) levels or without B symptom had better prognoses than those with high LDH level or with B symptom, respectively, in the group treated between 1995 and 1999, but not in those treated during 1985–1994.

To analyze the influence of treatment-related factors on outcome, patients who did not complete radiotherapy (and died soon) were excluded. In patients treated between 1985 and 1994, those who received partial-brain radiation, spinal radiation, or whole-brain dose < 40 Gy showed better prognoses, but these phenomena were not observed in patients treated between 1995 and 1999. Figure 2 shows survival curves according to the treatment modality, i.e., radiation alone vs radiation plus chemotherapy. In patients treated between 1985 and 1994, the two groups showed similar prognoses. In patients treated between 1995 and 1999, however, those who received radiation plus chemotherapy showed significantly better survival than those who received radiation alone. Among these patients, 61% of the

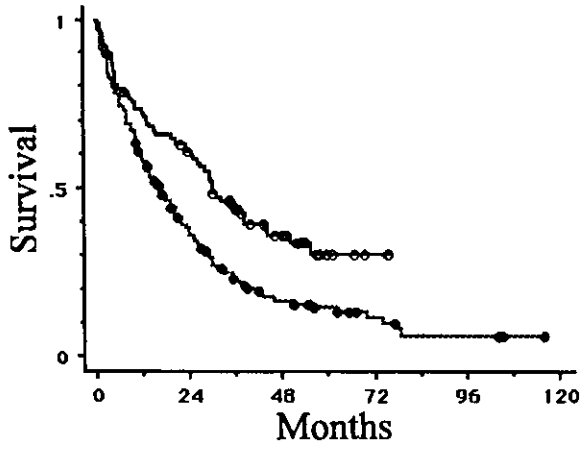

Fig. 1 Survival curves for patients with primary central nervous system lymphoma treated between 1985 and 1994 (- - -●- - -) and for those treated between 1995 and 1999 (____○____). The difference was significant ($P = 0.0003$)

patients who received radiochemotherapy were younger than 60 years, but 39% of those treated with radiation alone were younger than 60 years ($P = 0.050$). Similarly, 64% of the patients who received radiochemotherapy had a PS 0–2, but 55% of those treated with radiation had a PS 0–2 ($P = 0.50$). Figure 3 shows survival curves according to the chemotherapy regimens. In patients treated between 1985 and 1994, there was no significant difference in survival curves according to the regimens. On the other hand, there was an overall difference in those treated between 1995 and 1999 ($P = 0.018$). Patients receiving MTX-containing regimens showed better survival than those treated with CHOP/VEPA or COP ($P = 0.0071$).

Multivariate analyses were performed for potential prognostic factors, which were significant in univariate analyses (Table 4). Factors concerning the radiation field and spinal radiation were not included because of the small number of patients in one of the groups. In both patient groups treated during 1985–1994 and 1995–1999, age and tumor number were suggested to be significant prognostic factors. PS and LDH level did not reach statistical significance. The radiation dose to the whole brain and chemotherapy did not prove significant in patients treated between 1985 and 1994, and in those treated between 1995 and 1999, respectively.

Discussion

The most significant finding of this study appears to be that patients treated between 1995 and 1999 showed a significantly better prognosis than those treated between 1985 and 1994. Comparison of the patient and tumor characteristics revealed that there were more patients with better PS between 1995 and 1999 than between 1985 and 1994. This may be due to the earlier diagnosis of the disease in recent years and improvement in gen-

Table 3 Survival data according to potential prognostic factors (MST median survival time in months, 5-YSR 5-year survival rate)

Prognostic factor		1985–1994				1995–1999			
		n	MST	5-YSR(%)	*P*	*n*	MST	5-YSR(%)	*P*
Gender	Male	97	15	8.7	0.13	67	32	31	0.62
	Female	70	22	23		34	28	33	
Age (years)	< 60	83	20	22	0.0057	53	44	45	0.0052
	≥ 60	84	13	6.8		48	23	15	
Performance status	0–2	69	24	18	0.0015	60	37	32	0.024
	3,4	95	11	13		41	12	30	
B symptom	Yes	16	10	7.5	0.30	11	14	18	0.027
	No	133	18	17		81	36	35	
Lactate	Normal	49	22	31	0.17	50	55.5	43	0.0084
dehyrdogenase	High	34	21	5.8		30	20.5	(20)[b]	
Tumor number	Single	103	22	19	0.0021	56	55.5	43	0.0083
	Multiple	63	11	7.9		43	26	17	
Tumor size (cm)[a]	≤ 3 cm	–	–	–	–	51	32	33	0.95
	> 3 cm	–	–	–		41	37	31	
Radiation field	Whole brain	139	17	12	0.026	89	30	31	0.99
	Partial brain	19	35	38		8	35	(33)	
Spinal radiation	Yes	15	31	37	0.042	4	-	(50)	0.69
	No	143	17	13		93	30	30	
Total dose (Gy)	< 50	45	16	22	0.79	24	29.5	26	0.16
	≥ 50	113	18	13		73	36	32	
Whole-brain dose	< 40	61	24	22	0.025	38	32	26	0.83
(Gy)	≥40	97	14	11		59	30	32	
Chemotherapy	Yes	65	18	19	0.63	64	38	40	0.0049
	No	74	19	14		31	25	(14)	

[a] Maximum tumor diameter before radiation
[b] Figures in parentheses are 4-year survival rate

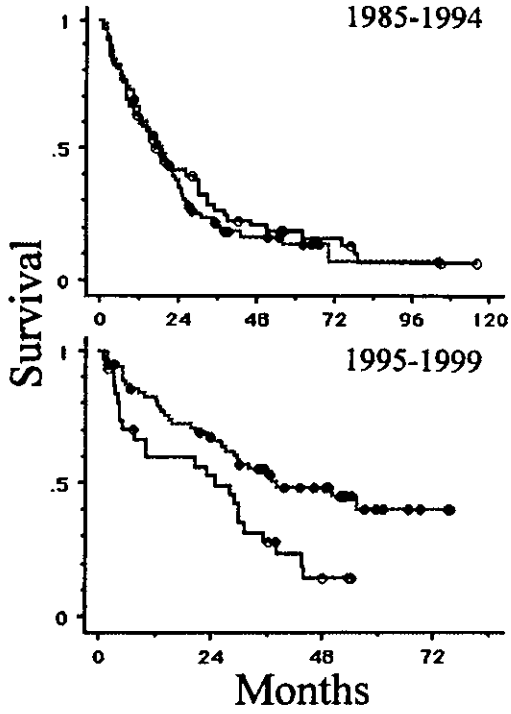

Fig. 2 Survival curves according to the treatment modality. ____O____ : patients treated with radiation alone, - - -●- - - : patients treated with radiation and chemotherapy. The difference was significant in the group of patients treated between 1995 and 1999 (upper panel, $P = 0.63$; lower panel, $P = 0.0049$)

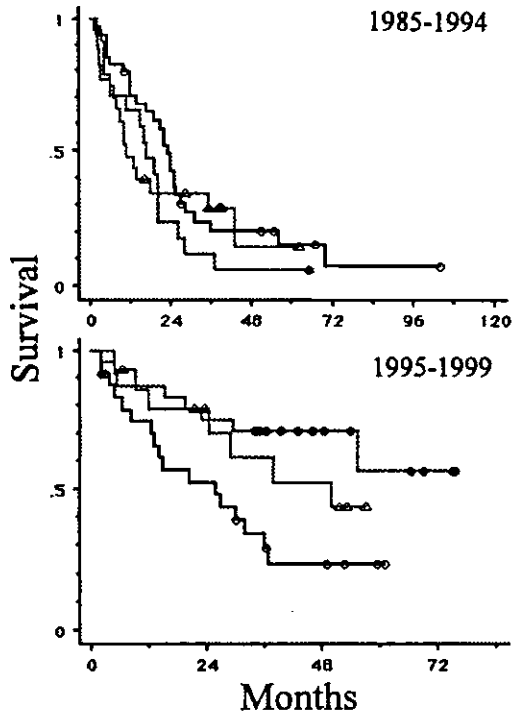

Fig. 3 Survival curves according to chemotherapy regimens. ____O____ : cyclophosphamide, vincristine, prednisolone ± doxorubicin, - - -●- - - : methotrexate-containing regimens, - - -Δ- - - : other regimens. The difference among the curves was significant in the group of patients treated between 1995 and 1999 (upper panel, $P = 0.32$; lower panel, $P = 0.018$)

Table 4 Multivariate anayses for potential prognostic factors that were significant in univariate analysis

Factor	1985–1994 (n = 154)		1995–1999 (n = 72)	
	P	Relative risk	P	Relative risk
Age (< 60 vs ≥ 60 years)	0.036	1.48 (1.03–2.15)[a]	0.047	2.07 (1.01–4.22)
Performance status (0–2 vs 3,4)	0.13	1.36 (0.92–2.01)	0.13	1.77 (0.85–3.68)
Lactate dehydrogenase (normal vs high)	–	–	0.13	1.70 (0.86–3.34)
Tumor number (single vs multiple)	0.0093	1.67 (1.13–2.45)	0.0032	2.82 (1.42–5.62)
Whole-brain dose (< 40 vs ≥ 40 Gy)	0.22	1.28 (0.86–1.91)	–	–
Chemotherapy (yes vs no)	–	–	0.23	1.53 (0.32–1.31)

[a]Figures in parentheses are 95% confidence intervals

eral care including corticosteroid therapy and less aggressive surgery. Since PS was a significant prognostic factor in univariate analysis, it is suggested that the increase in the proportion of better PS patients may, at least in part, have contributed to the improvement in prognosis in patients treated between 1995 and 1999.

Age, PS, and tumor multiplicity are well-known prognostic factors for PCNSL (Corry et al. 1998; Hayabuchi et al. 1998; O'Brien et al. 2000). The present results of univariate analyses agree with these previous observations, although the influence of PS did not reach a significant level in multivariate analysis. Patients with a high LDH level treated between 1995 and 1999 showed a poorer prognosis than those with a normal LDH level in univariate analysis. However, LDH was not a significant factor in patients treated between 1985 and 1994, as also shown in the multivariate analysis of patients treated between 1995 and 1999. The previous analysis of 466 patients in the nationwide survey suggested an association of high LDH level and poor prognosis in both univariate and multivariate analyses (Hayabuchi et al. 1998), so LDH may be a potential prognostic factor which is certainly weaker than age, PS, and tumor multiplicity. A similar finding was obtained regarding B symptom. In the newer survey, we investigated the influence of tumor size, but it did not appear to have a significant influence on patient outcome.

Regarding the method of radiation therapy, patients who were treated with a partial-brain field showed a better prognosis than those treated with a whole-brain field in the group treated between 1985 and 1994. Shibamoto et al. (Shibamoto et al. 2003) recently discussed the possible benefit of using partial-brain irradiation, especially in patients with a single lesion. Due to the retrospective nature of the present study and the small number of patients who received partial-brain irradiation, no conclusion should be drawn regarding radiation field, but avoiding whole-brain radiation may be a future topic in the treatment of PCNSL. The observation in the earlier period that patients who received spinal radiation and those who received whole-brain doses of less than 40 Gy had a better prognosis are paradoxical, and it is suggested that these observations would represent patient selection bias, which is often seen in retrospective analysis. As has been suggested by previous findings (Nelson et al. 1992; Hayabuchi et al. 1998), a higher dose of radiation did not appear to be associated with survival improvement.

In patients treated between 1985 and 1994, those who received radiation alone and those who received radiation plus chemotherapy showed a similar prognosis. On the other hand, in patients treated between 1995 and 1999, those who received radiation plus chemotherapy had a significantly better prognosis than those who received radiation alone. However, the effect of chemotherapy was not significant in multivariate analysis. Since younger patients were more often treated with combined radiation and chemotherapy, this may be one of the reasons why the effect of chemotherapy was not supported by multivariate analysis. Analysis according to chemotherapy regimens suggested a possible advantage of MTX-containing regimens over conventional CHOP or similar regimens. Several studies have suggested the ineffectiveness of CHOP or similar regimens, especially when given before radiation (Schultz et al. 1996; O'Neill 1999; Mead et al. 2000), although post-radiation CHOP requires further investigation (Shibamoto et al. 1999). The present findings suggest that systemic chemotherapy with weak or moderate intensity may not be beneficial in PCNSL.

The findings of the present study revealed that the treatment outcome for PCNSL varies greatly with the era. Although most of the chemotherapy regimens used were of mild or moderate intensity and only 14% of the patients received high-dose-MTX-containing chemotherapy, the 5-year survival rate of 31% for all patients treated between 1995 and 1999 (including those who did not complete radiotherapy) were equal to that recently reported by the Radiation Therapy Oncology Group (DeAngelis et al. 2002) or those of other series using intensive combined modality treatment including high-dose MTX (Brada 1998; Bessell et al. 2001). Therefore, it appears to be inappropriate to discuss the usefulness of treatment modality by comparing with the historical control data. There have been no major randomized studies, except for a small one (Mead et al. 2000), regarding the benefit of combining chemotherapy with radiation, but to confirm the efficacy of chemotherapy, randomized studies appear to be necessary.

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Topotecan and Irinotecan in the Treatment of Pediatric Solid Tumors

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Abstract: There have been significant advances in the treatment of neuroblastoma and rhabdomyosarcoma, but the clinical results are still poor, especially after tumor relapse. In addition to this, rhabdomyosarcoma does worse if localized tumors occur in unfavorable sites. Therefore, new chemotherapeutic agents have been sought, and the effects of 9-dimethylaminomethyl-10-hydroxycampthothecin (topotecan) and 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyl-camptothecin hydrochloride (irinotecan) were studied preclinically and clinically during the past decade not only in adults but also in children. Irinotecan and topotecan inhibit DNA topoisomerase I, which is an essential nuclear enzyme that relaxes torsionally strained duplex DNA, enabling replication and transcription. These agents were reported to be effective against various human malignancies in adults. Among these camptothecin derivatives, topotecan and irinotecan are the most widely used clinically, and at present irinotecan appears to be more promising in the treatment of childhood solid tumors such as rhabdomyosarcoma and neuroblastoma. The recommended dose and administration schedule differ among clinical trials.. For example, 1-day, 3-day, and 10-day regimens have been used. In the present article, the clinical effectiveness of topotecan and irinotecan with different administration schedules are reviewed in the US, French and Japanese literature, and the authors propose which agent and which administration schedule of these agents are the most effective in the treatment of pediatric solid tumors.

Keywords: Topotecan, irinotecan, neuroblastoma, rhabdomyosarcoma, leiomyosarcoma, phase-II trials.

DEVELOPMENT OF TOPOTECAN AND IRINOTECAN

Significant advances in survival rates have been achieved in the treatment of several pediatric solid tumors such as advanced neuroblastoma [1,2] and rhabdomyosarcoma [3,4], but the clinical results are still unsatisfactory, especially in patients with disseminated disease. Therefore, numerous new agents such as paclitaxel, fotemustine, busulfan, mitomycin C, ifosfamide, and bleomycin have been investigated for their efficacy in preclinical studies [5-7], and only a few were found to be sufficiently promising to be incorporated in clinical trials. The topoisomerase I inhibitors topotecan and irinotecan are examples of such promising agents.

In the Yangtze River basin of China, elderly Chinese are aware that leaves of the tree *Camptotheca acuminata* are effective against human gastric cancer. The antitumor activity of 20(S)-camptothecin, a plant alkaloid isolated from *C. acuminata,* was first studied more than 20 years ago [8]. Although 20(S)-camptothecin is insoluble in aqueous vehicles, extensive investigation has identified more soluble and active camptothecin analogs. The water-soluble analog, 9-dimethylaminomethyl-10-hydroxy-camptothecin (topotecan) demonstrate sbroad-spectrum activity against rodent tumor models [9] and significant therapeutic activity against some human colon adenocarcinoma xenografts [10].

Another water-soluble derivative of camptothecin, 7-ethyl-10-(4-[1-piperidino]-1-piperidino)-carbonyl-camptothecin hydrochloride trihydrate (irinotecan, CPT-11), was synthesized [11]. Irinotecan and topotecan [12] inhibit DNA topoisomerase I, which is an essential nuclear enzyme that relaxes torsionally strained duplex DNA, enabling replication and transcription. However, irinotecan is a masked compound and, as opposed to topotecan, the hydrolysis of the piperidino-piperidine side-chain by carboxylesterase leads to the formation of the active metabolite SN-38 [13]. *In vitro* studies have demonstrated that SN-38 is the most potent active drug among the topoisomerase I inhibitors so far available for clinical development [14].

PRECLINICAL STUDIES OF TOPOTECAN

Studies in mice bearing human solid tumors have shown that topotecan is among the most active anticancer drugs. Significant objective responses have been obtained in colon carcinoma [10,15], rhabdomyosarcoma [10,15,16], neuroblastoma [17], osteosarcoma [10], and brain tumors [15,16]. In addition, studies in these xenograft models have shown that antitumor activity is highly dependent on the administration schedule, including dosage. Responses were frequently observed when the agent was given daily at low doses for protracted periods [15,17]. In some studies, prolonged administration induced responses in xenografted tumors that had been unresponsive to intermittent administration of the agents at high doses [15].

CLINICAL STUDIES OF TOPOTECAN

Phase I Studies

Phase I studies of topotecan have investigated various administration schedules and doses. Based on the S-phase specificity of topotecan, most phase I trials explored

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schedules that result in prolonged exposure to maximize the drug's interaction with topoisomerase I during the S-phase. The regimens investigated included, for example, one 24-h infusion every 3 weeks, continuous 72- to 120-h infusion every week or every other week, and 30-min infusion for 5 days every 3 weeks [18,19]. The results of those studies suggested that prolonged exposure induces more responses than short-term exposure at higher concentrations. The regimen recommended for most phase II studies is 1.5 mg/m^2 daily for 5 days every 3 weeks [20]. With these schedules and doses, the dose-limiting toxicity was found to be myelosuppression [20].

In children, phase I studies have also explored different schedules of prolonged administration (for example, 72-h infusion [12], 120-h infusion [21], or daily × 5 [22]). The dose-limiting toxicity in most pediatric trials was also myelosuppression, and the maximum tolerated dose (MTD) is 1.4 mg/m^2 per day for 5 days, if granulocyte-colony-stimulating factor is not given [22]. A 10-day topotecan administration schedule ([qd × 5] × 2) was also successful [23,24].

Phase II Studies

Most adult phase II studies of topotecan given as a single agent administered 1.5 mg/m^2 daily for 5 days every 3 weeks (the most effective schedule in phase I) to patients with advanced or recurrent disease [20]. In adults, many patients with lung cancer or other advanced disease are enrolled in phase II from the beginning even though they are previously untreated [20]. In randomized trials, this schedule has yielded better responses than schedules of intermittent administration [25]. The best responses have been obtained in patients with advanced ovarian carcinoma [25-31] and in patients with advanced lung cancer [32-36]. Minimal responses have been observed in patients with colorectal cancer and gastric cancer [20]. Finally, as many as 43% of previously untreated patients with myelodysplastic syndrome (MDS) or chronic myelomonocytic leukemia (CMML) showed complete responses to topotecan given by continuous infusion for 5 days [37]. These responses were associated with the disappearance of genetic abnormalities characteristic of MDS/CMML [37].

There have been two phase II studies of topotecan in children. Blaney and co-workers administered topotecan as a 72-h continuous infusion at a dosage of 1 mg/m^2/day to children with refractory neuroblastoma or sarcomas [38]. The antitumor activity was minimal on this schedule, as responses were obtained in only 1 of 26 patients with neuroblastoma and in only 1 of 25 patients with primitive neuroectodermal tumor (PNET) [38]. A protracted schedule was used in a Pediatric Oncology Group Study in which children with refractory solid tumors were given 2 mg/m^2/day for 5 days. The dose administered was also greater in the latter schedule, and complete (CR) and partial responses (PR) were observed in patients with neuroblastoma, PNET, and retinoblastoma [38,39]. Two up-front window studies used the same dosage and schedule. A Pediatric Oncology Group study obtained objective responses in 38% of children with stage IV neuroblastoma [40]. An Intergroup Rhabdomyosarcoma Study Group phase II study in previously untreated patients with rhabdomyosarcoma obtained an overall response rate of 45% (67% for alveolar rhabdomyosarcoma) [41]. These results confirmed that topotecan given on a protracted schedule has a definite role in the treatment of childhood solid tumors such as neuroblastoma, PNET, and rhabdomyosarcoma.

PRECLINICAL STUDIES OF IRINOTECAN

Irinotecan, a water-soluble prodrug, has been shown to have broad-spectrum activity against experimental adult and pediatric tumor models [15,42-48]. Significant objective responses to irinotecan have been observed in pediatric rhabdomyosarcoma [10,15,16,48], neuroblastoma [13,17,49-51], brain tumors [15,16,52], and osteosarcoma [10]. In particular, rhabdomyosarcoma and neuroblastoma showed irinotecan sensitivity, and in the history of pediatric oncology, this is the first time that the clinical development of a new anticancer drug started with specific preclinical data obtained from pediatric tumor models. Irinotecan is clearly very active against pediatric tumor xenografts. Can these preclinical results predict the efficacy of irinotecan in children with solid tumors? Irinotecan is biotransformed *in vivo* to its active metabolite SN-38 by carboxylesterase. Murine plasma contains carboxylesterase, while human plasma does not [13]. Therefore, phase I and II clinical trials were particularly needed.

CLINICAL STUDIES OF IRINOTECAN

Phase I Studies

Most of the phase I studies of irinotecan in adults investigated 90-min infusions administered every week for 3 to 4 weeks or once every 3 weeks [53,54].

Four phase I trials of irinotecan in children were conducted in the USA, France, and Japan [55-58]. Furman and co-workers [55] recommended administration of irinotecan 20 mg/m^2/day for 5 consecutive days, repeated once after 2 days off (10-day administration in total) based upon their results of irinotecan experiments in an *in vivo* system. Similarly, Blaney *et al.* studied the administration of irinotecan for 5 consecutive days, repeated every 3 weeks [56]. On the other hand, Vassal and co-workers [57] reported that the MTD of irinotecan in children was 600 mg/m^2 when given as a 120-min intravenous infusion every 21 days. Mugishima *et al.* [58] determined that the MTD of irinotecan for children was between 160 mg/m^2/day and 180 mg/m^2/day administered over 3 consecutive days, repeated once after 25 days off. The MTDs obtained in those studies are currently recommended for phase II trials by the respective study groups (Table 1).

Phase II Studies

A variety of phase II trials of irinotecan have been conducted and this agent has been reported to be effective against various adult human malignancies, including lymphoma, gastric cancer, small cell lung cancer, non-small cell lung cancer, cervical cancer, epithelial ovarian cancer, and colorectal cancer [59-65].

The doses and administration schedules, currently recommended for phase II trials of irinotecan in children

Table 1. Recommended Administration Schedules for Phase II Trials of Irinotecan in Children and Their Tentative Clinical Results.

Phase I Trials		Phase II Trials
Authors	**Recommended Administration Schedules for Phase II**	**Remarks**
Mugishima *et al.* [58] (Japan)	180 mg/m^2/day for 3 consecutive days; repeated every 4 weeks	On-going
Furman *et al.* [55] (USA)	20 mg/m^2/day for 5 consecutive days repeated once after 2 days off; repeated every 4 weeks	21% response rate; 2 CR/2 PR in 19 patients (Cosetti *et al.*[66])
Blaney *et al.*[56] (USA)	40 mg/m^2/day for 5 consecutive days; repeated every 4 weeks	On-going
Vassal *et al.*[57] (France)	600 mg/m^2/day for one day; repeated every 3 weeks	Disappointing in neuroblastoma (Vassal *et al.*[67])

(Table 1), have merits and demerits [55-58,66]. Cosetti *et al.* [66] saw 4 objective responses (2 CR and 2PR) (21.1% response rate) in 19 valuable patients treated on the administration schedules developed by Furman *et al.* [55]. Protracted use (intravenous or oral) of irinotecan might be recommended because of its consistent effectiveness, but the use of irinotecan over 12 days could be somewhat burdensome for patients and clinicians. On the other hand, the single-day administration of irinotecan yielded disappointing clinical results in phase II trials in relapsed neuroblastomas [57], and therefore Vassal *et al.* concluded that their dosing schedule of irinotecan [57] showed no clinically useful activity. Vassal *et al.* further mentioned that because the majority of children had received very intensive induction treatment and retinoids, it was unlikely that a single agent in a phase II setting would demonstrate activity [67]. They considered that they needed to evaluate neuroblastoma in a different setting in the future to prevent clinically important agents from being missed [67].

Cosetti *et al.* [66] observed CR or PR in 3 of 4 patients with relapsed rhabdomyosarcoma on the irinotecan administration schedule of Furman *et al.* [55], and this observation coincides with Shitara *et al.*'s results in a rhabdomyosarcoma trial even though they used a different administration schedule [68]. Shitara and collaborators reported that administration over 3 consecutive days may have an advantage over other schedules. They administered irinotecan 180 mg/m^2/day for 3 consecutive days having already confirmed protracted plasma concentrations of irinotecan with their 3-day administration schedule [68]. With this administration schedule, which differs from those in the USA and France, PR was observed in 38.5% of the relapsed/refractory patients, with acceptable toxicities [68]. The PR was achieved in leiomyosarcoma, rhabdomyosarcoma, neuroblastoma [69], undifferentiated sarcoma, and Wilms' tumor (Table 2). As a single, independent experience, Rosoff and Bayliff [70] administered irinotecan 50 mg/m^2/day for 5 days every 3–4 weeks in 2 patients with desmoplastic round blue cell tumors and saw significant responses. As far as the treatment of childhood solid tumors is concerned, at present irinotecan appears to be promising in the treatment of childhood solid tumors such as rhabdomyosarcoma, neuroblastoma, and desmoplastic round blue cell tumor [55,58,66,68-70].

ORAL ADMINISTRATION OF TOPOTECAN AND IRINOTECAN

The efficacy of the protracted oral administration of topotecan and irinotecan has been well established *in vivo* [15,50,71]. While previous studies [72-74] evaluated the safety and disposition of oral topotecan in adults, the oral administration of topotecan in children has also recently been evaluated in a phase I study [75]. In addition, Daw *et al.* found that the MTD was 1.8 mg/m^2/day on a daily × 5 × 2 schedule, which was higher than the MTD for adults [74], and that the disease stabilized in 9 of 19 assessable patients for 1.5 to 6 months [75]. The dose-limiting factors were myelosuppression and diarrhea in this pediatric cohort receiving oral topotecan [75]. However, oral administration of irinotecan has not yet undergone phase I trials in pediatric patients with malignant solid tumors.

TOPOISOMERASE I INHIBITORS GIVEN IN COMBINATION WITH OTHER CHEMOTHERAPEUTIC AGENTS

It has been suggested that topoisomerase I inhibitors should be investigated in combination with other chemotherapeutic agents [20]. There is evidence of a lack of cross-resistance between camptothecin analogues and other anticancer drugs [76]. Furthermore, several preclinical studies showed that the administration of topoisomerase I inhibitors with alkylators, platinum compounds, topoisomerse II inhibitors, and antimicrotubule agents produces additive or synergic antitumor activity [77,78]. However, an antagonistic rather than a synergic effect may also be produced by camptothecin analogs when combined with certain drugs [79,80]. The efficacy of drug combinations depends on the schedule of administration and on the choice of drugs. Several adult and pediatric clinical

Table 2. Results of Phase II Administration* of Irinotecan Studied by the Authors' Group [68]

Disease	No. of Patients	PR	SD**	SD	PD
Leiomyosarcoma	1	1			
Neuroblastoma	6	1	2	2	1
PNET	1				1
Undifferentiated sarcoma	1	1			
Wilms' tumor	2	1			1
Rhabdomyosarcoma	2	1			1
Total	13	5	2	2	4

*, irinotecan 180 mg/m^2/day for 3 consecutive days, repeated once after 25 days off; PR, partial response; SD**, stable disease (SD) but with transient decrease in tumor marker levels; PD, progressive disease; PNET, primitive neuroectodermal tumor.

studies (phase I and phase II) of multiagent therapy including topotecan and irinotecan were reviewed by Rodriques-Galindo *et al.* [20].

Noda and associates [81] recently compared clinical results of patients treated with irinotecan plus cisplatin and those treated with etoposide plus cisplatin for extensive small-cell lung cancer. At the time when the 154 patients were enrolled, the median survival was 12.8 months in the irinotecan / cisplatin group and 9.4 months in the etoposide / cisplatin group (p=0.002 by the unadjusted log-rank test). At 2 years, the proportion of patients surviving was 19.5% in the irinotecan/ cisplatin group and 5.2% in the etoposide / cisplatin group [81]. Severe or life-threatening myelo-suppression was more frequent in the etoposide-/ cisplatin group than in the irinotecan/ cisplatin group, and severe or life-threatening diarrhea was more frequent in the irinotecan/cisplatin group than in the etoposide / cisplatin group.

In children, cyclophosphamide plus topotecan was administered to patients with recurrent or refractory solid tumors [82], and the combination of cyclophosphamide and topotecan was found to be active against rhabdomyosarcoma, neuroblastoma, and Ewing's sarcoma [82]. Similarly, a window trial with topotecan and cyclophosphamide carried out in children with newly diagnosed metastatic rhabdomyosarcoma [41] found that topotecan after cyclophosphamide is active against newly diagnosed rhabdomyosarcoma. However, survival rates remained disappointing for children with metastatic rhabdomyosarcoma at diagnosis [41]. The problems related to the combined use of topotecan with cyclophosphamide was extensively reviewed [41].

There are many combinations of topoisomerase I inhibitors with other chemotherapeutic agents in adults [20], but a combination of topotecan/irinotecan with the alkylating agent cyclophosphamide has been employed more frequently in children [20,41,82,83]. Kushner and his coworkers [83] used such a combination in the treatment of resistant neuroblastoma.

POLYMORPHISMS OF THE URIDINE-DIPHOSPHATE-GLUCURONOSYLTRANSFERASE GENE AND IRINOTECAN TOXICITY

Irinotecan unexpectedly causes severe toxicity in the form of leukopenia or diarrhea, presumably because it is metabolized to form active SN-38, which is further conjugated and detoxified by the uridine diphosphate (UDP)-glucuronosyltransferase (UGT) 1A1 enzyme [84]. Genetic polymorphisms of UGT1A1 would affect the interindividual variation in irinotecan toxicity via the alteration of the bioavailability of SN-38. Ando and co-workers studied the relationship between multiple variant genotypes (UGT1 A1*28 in promoter and UGT1A1*6, UGT1A1*27, UGT1A1*29, and UGT1A1*7 in the coding region) and severe toxicity of grade 4 leukopenia and/or grade 3 or 4 diarrhea in patients with various cancers [84]. Of 26 patients with severe toxicity, the genotypes of UGT1A1*28 were homozygous in 4 (15%) and heterozygous in 8 (31%), whereas 3 (3%) were homozygous and 10 (11%) heterozygous among the 92 patients without severe toxicity. Multivariate analysis suggested that the genotype either heterozygous or homozygous for UGT1A1*28 would be a significant risk factor for severe irinotecan toxicity [84].

Font *et al.* [85] similarly investigated the relationship with toxicity and the antitumor activity in patients with non-small cell lung cancer, but found no differences in toxicity based on UGT1A1 polymorphism. They also concluded that the tendency for a better prognosis in patients carrying the variant genotype 6/7 and 7/7 of the UGT1A1 gene requires further validation [85].

SUMMARY

The advent of topotecan and irinotecan in the treatment of childhood solid tumors may be comparable to that of cisplatinum, which occurred some 20 years ago. Topotecan was utilized first, and subsequently irinotecan has been employed pre-clinically and clinically. Unfortunately, these two agents were not fully investigated in phase I and II trials. The clinical application of such new agents requires the