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IV. 代表的論文

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The Japanese cord blood bank network experience with cord blood transplantation from unrelated donors for haematological malignancies: an evaluation of graft-versus-host disease prophylaxis

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Summary. Cryopreserved umbilical cord blood (CB) from unrelated donors can restore haematopoiesis after myeloablative therapy in patients with haematological malignancy. We investigated the clinical outcomes of CB transplantation (CBT) with special emphasis on graft-versus-host disease (GVHD) prophylaxis. Patients with haematological malignancies ($n = 216$) received intensive chemotherapy or immunosuppressive therapy, followed by transplantation of cryopreserved CB cells from unrelated donors. The clinical outcomes, i.e. haematological reconstitution, the incidence of acute or chronic GVHD, relapse and event-free survival (EFS), were evaluated. The estimated probability of neutrophil recovery was 88·2%. The median follow-up for the survivors was 557 d (range 21–1492 d). The overall and EFS rates were 32·6% and 25·5%, respectively, 3·5 years after transplantation. Multivariate analysis using Cox's proportional hazards model showed that high-risk disease status at CBT and single-drug GVHD prophylaxis were associated with worse 2-year EFS rates [$P = 0·0013$, relative risk (RR) 1·90, 95% confidence interval (CI) 1·28–2·81 and $P = 0·0007$, RR 1·91, 95% CI 1·31–2·79 respectively). Age at CBT had no significant influence on EFS. Cryopreserved CB from unrelated donors can restore haematopoiesis in patients with haematological malignancy. Although the incidence is low, the prophylaxis for acute GVHD is an important factor for survival of CBT from unrelated donors. A high rate of suitable donors was found, with a probability of 1 to every 18 CB units, when compared with human leucocyte antigen matching at other haematopoietic stem cell banks.

Keywords: cord blood transplantation, unrelated donor, GVHD prophylaxis, haematological malignancy, Japanese cord blood bank network.

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Haematopoietic stem cell transplantation (HSCT), using cells from a human leucocyte antigen (HLA)-matched sibling or unrelated bone marrow (UBM) donors, has been successful in treating patients with high-risk or relapsed haematological malignancies, syndromes of bone marrow failure, hereditary immunodeficiencies and

metabolic disorders (Thomas *et al*, 1999). To potentially expand the donor pool, placental umbilical cord blood (CB) has been used as an alternative source of HSCs. Approximately 1500 CB transplants have been performed worldwide since the first transplantation of CB from unrelated donors in 1993 (Wagner *et al*, 1995, 1996; Kurtzberg *et al*, 1996; Gluckman *et al*, 1997; Rubinstein *et al*, 1998; Gluckman, 2000; Barker *et al*, 2001; Laughlin *et al*, 2001; Ohnuma *et al*, 2001; Rocha *et al*, 2001; Sanz *et al*, 2001). For patients who require HSCT but for whom there are no suitable related donors available, this source of HSC offers substantial advantages: prompt availability of cryopreserved donor cells and less stringent requirements of HLA typing between the donor and recipient because of low risk of inducing a severe graft-versus-host disease (GVHD). The absence of or a reduction in the component of graft-versus-tumour (GVT) effect associated with GVHD, on the other hand, may represent a theoretical concern for leukaemic patients who have received cord blood transplantation (CBT).

The Japanese Cord Blood Bank Network (JCBBN) was established by the Ministry of Health, Labour and Welfare in August 1999. Nine domestic cord blood banks are affiliated to the network, which provides support to those patients who require HSCT. Suitable donors are identified from approximately 7000 available units, effectively relying on the Internet. The results of CBT with cryopreserved HLA-matched or HLA-mismatched unrelated donors in 216 patients with haematological malignancies are reported. The analysis of HLA disparities and disease-related factors that affect the event-free survival, the incidence of acute GVHD, GVHD prophylaxis, engraftment, relapse and transplantation-related mortality (TRM) of the recipients of CBT from unrelated donors were considered.

PATIENTS AND METHODS

Patients. Between February 1997 and August 2001, 216 unrelated donor–recipient pairs underwent CBT. All the recipients suffered from haematological malignancies (Table 1) and had a median age of 6·0 years with a median body weight of 20·0 kg (Table I). Other characteristics and the disease status of leukaemia patients undergoing unrelated CBT are described in Table I. It has been reported that for patients with leukaemia and myelodysplastic syndrome (MDS), the disease status is the main factor influencing the transplant outcome (Balduzzi *et al*, 1995; Casper *et al*, 1995; Locatelli *et al*, 1999; Uderzo *et al*, 2000). Therefore, patients receiving transplants during the first or second complete remission (CR), MDS without blasts, familial haemophagocytic lymphoproliferative syndrome (FHL) or non-Hodgkin's lymphoma (NHL) during first remission, were assigned to the standard-risk (SR) group ($n = 93$). Those in their third or subsequent remission, relapse or partial remission with refractory leukaemia at the time of CBT, were considered to be in the advanced phase and were placed in the high-risk (HR) group ($n = 86$). Patients with blast crisis of chronic myeloid leukaemia (CML) or second CR after relapse of HSCT in the first CR were included in the high-risk group ($n = 123$).

Table I. Demographic characteristic of 216 CBT patients.

Sex	
Male	121
Female	95
Age at transplant (years)	
median	6
range	0–56
Body weight at transplant (kg)	
median	20
range	4·2–75
≤ 10	41
11–20	69
21–30	29
31–40	23
41–50	27
> 50	27
Infused cells($\times 10^7$/kg)	
median	3·6
range	0·5–19·5
HLA disparity, serological	
6/6	27
5/6	134
4/6	55
GVHD prophylaxis	
One drug	
CsA	45
FK506	10
MTX	7
Pred	1
Two drugs	
FK + MTX	4
CsA + MTX	65
FK + Pred	10
CsA + Pred	38
Diagnosis	
Acute lymphoblastic leukaemia	104
Acute myeloid leukaemia	71
Chronic myeloid leukaemia	5
Malignant lymphoma	11
Myelodysplastic syndrome	22
FHL	3

FHL, familiar haemophagocytic lymphoproliferative syndrome; FK506, tacrolimus; CsA, cyclosporin A; MTX, methotrexate; Pred, prednisolone.

Eligibility. The clinical protocols for the CBT were approved by the institutional review board of each participating institution. Patients were eligible for enrolment if their disease was stable but had no HLA-identical related or unrelated donor, or if their disease was unstable with no related donors and an HLA-matched unrelated bone marrow donor could not be identified within 6–8 weeks. The disease status of those with haematological malignancies was categorized according to the criteria specified in the International Bone Marrow Registry (Sobocinski *et al*, 1994).

Donor registries and selection of grafts. Searches for unrelated CB donors were processed through the JCBBN where 4860 CB units were available in August 2001. A preliminary search of CB banks was performed using the patient's HLA phenotype, as determined by serological typing for class I HLA-A and HLA-B antigens, and high-resolution DNA typing for class II HLA-DRB1 alleles. Preferred CB units were those matched at four or more of six HLA loci and that contained a minimum cell count of 2×10^7 nucleated cells/kg of the recipient's body weight before freezing. The HLA disparity of HLA-A, B and DR of recipients and graft is shown in Table I. CB units were not depleted of T lymphocytes.

Preparative regimen and prophylaxis against GVHD. The conditioning regimen and acute GVHD prophylaxis varied according to the centre policy, type of disease, prior treatment and disease status at the time of an unrelated CBT. The patients or their parents gave consent to the CBT after being informed of the potential risks and benefits of the procedure. The GVHD prophylaxis is summarized in Table I. Supportive therapy differed among the transplant centres. Protocols for intensive myeloablative therapy and the use of CB from unrelated donors for transplantation were reviewed and approved by the institutional review boards at the transplant centres.

Follow-up and end points. The status of all patients was evaluated based on the last follow-up report (31 August 2001); thus, we were able to achieve a 100% rate of follow-up. Chimaerism was evaluated by fluorescent *in situ* hybridization for the Y chromosome in sex-mismatched grafts and DRB1 allele-specific hybridization or quantitative polymerase chain reaction analysis for microsatellite DNA markers in sex-matched transplantations. The time to neutrophil recovery was defined as the first of 3 d consecutively after transplantation during which the absolute neutrophil count (ANC) was at least $0{\cdot}5 \times 10^9$/l. The time to platelet recovery was defined as the first 7 d in which the platelet count was at least 50×10^9/l without transfusion support. Primary graft failure was defined as a failure to reach a an ANC of $0{\cdot}5 \times 10^9$/l within 42 d of transplantation or a continued need for platelet transfusions for more than 60 d after transplantation in the absence of a leukaemic relapse. Those receiving a second transplant following non-engraftment were checked at the time of the second transplant. Patients were considered at risk of developing acute GVHD from d 1 post transplant. Those with sustained engraftment of donor haematopoietic cells who survived for more than 100 d post transplant were evaluated for the development of chronic GVHD. Acute and chronic GVHD were evaluated according to standard criteria (Shulman *et al*, 1980; Przepiorka *et al*, 1995). Relapse was indicated by the morphology of leukaemic cells in the bone marrow, peripheral blood or cerebrospinal fluid, or by the cytogenetic assay of a malignant clone. The time interval between CBT and relapse was recorded at examinations, while confirming the patient's remission status. Transplantation-related mortality (TRM) was defined as all causes of non-leukaemic deaths within 1 year of transplant. Overall survival (OS) was defined as the time interval between CBT and death due to any cause. Event-free survival (EFS) was defined as the time between CBT and a first event, which included graft failure, relapse or death during CR.

Statistical analysis. The investigators submitted primary data and annual follow-up reports to the data centre for the Programme for CBT in Japan. Transplantation procedures were performed at 57 institutes throughout the country. The results were analysed as of 31 August 2001. The median duration of follow-up was 557 d (range 21–1492 d). No patients were lost to follow-up by 31 August 2001, the day on which all centres verified their data on transplant characteristics and each patient's outcome. Categorical data in cross-tabulation tables were compared by using the Fisher's exact test. OS, EFS, acute GVHD, relapse and TRM after CBT (starting-point interval) were evaluated by employing the Kaplan–Meier product limit method and compared by using the log-rank test (Kaplan & Meier, 1958). All variables that were found to have a *P*-value of less than 0·1 by the log-rank test were included as binary covariates in a Cox's proportional hazards model (Cox, 1972). Relative risk (RR) for the association between covariates and events was estimated from Cox's model. All statistical analyses were carried out with STATVIEW version 5·0 software (SAS Institute, Cary, NC, USA).

RESULTS

Neutrophil recovery

Graft failure occurred in 23 (10·5%) of 216 patients. Of those who received transplants from unrelated donors, 88·1% patients exhibited neutrophil recovery on d 42 after transplantation by Kaplan–Meier estimates. The median time to neutrophil recovery was 24 d (range 11–219 d). Details on the univariate analysis for neutrophil recovery are shown in Table II. In multivariate analysis using Cox's proportional hazards model, the significant factors influencing neutrophil engraftment were: the number of cells infused [greater than $3{\cdot}6 \times 10^7$/kg [$P = 0{\cdot}0003$, RR 2·17, 95% confidence interval (CI) 1·43–3·31)], HLA serologically fully matched antigen ($P = 0{\cdot}01$, RR 1·99, 95% CI 1·12–3·51) and HLA serological one-locus antigen mismatch ($P = 0{\cdot}009$, RR 1·74, 95%CI 1·14–2·66).

Platelet recovery

The median time to achieve a platelet count $\geq 50 \times 10^9$/l was 47 d (range 10–962 d). The probability of platelet recovery by d 60 after transplantation in these patient groups was 61·9%. Details on the univariate analysis for platelet recovery are shown in Table II. From the Cox model, the favourable factor for platelet recovery was GVHD prophylaxis using a single drug ($P = 0{\cdot}03$, RR 1·55, 95% CI 1·02–2·36).

GVHD

Of the 193 patients who were available for evaluation and had myeloid engraftment, 149 (68·9%) developed acute GVHD, 25 died from complications within 28 d after CBT without myeloid recovery, 20 were not available for

Table II. Univariate analysis using the log-rank test for haematological recovery, EFS, relapse rate and TRM rate.

	Neutrophil recovery		Platelet recovery		2-year EFS		Relapse		TRM	
	% ± SE	*P*-value	% ± SE	*P*-value	% ± SE	*P*-value	% ± SE	*P*-value	% ± SE	*P*-value
Patient age (years)										
≤ 6	89·8 ± 3·1	0·05	70·8 ± 4·6	0·01	31·0 ± 5·4	0·107	48·7 ± 7·0	0·77	46·7 ± 5·8	0·0068
> 6	86·4 ± 3·6		51·8 ± 5·4		19·4 ± 4·7		56·9 ± 9·6		66·9 ± 5·4	
Sex										
M	82·4 ± 3·7	0·008	57·3 ± 4·9	0·25	23·0 ± 4·7	0·65	60·3 ± 8·0	0·14	59·5 ± 5·4	0·98
F	96·1 ± 2·2		69·7 ± 5·3		28·8 ± 5·3		40·6 ± 7·5		54·6 ± 5·9	
Body weight (kg)										
< 20	87·8 ± 3·3	0·33	70·5 ± 4·7	0·02	28·2 ± 5·1	0·37	52·5 ± 7·0	0·37	47·6 ± 5·7	0·015
≥ 20	88·6 ± 3·4		52·3 ± 5·4		22·4 ± 5·0		51·1 ± 9·8		66·2 ± 5·4	
Risk										
High	84·7 ± 3·6	0·02	58·3 ± 5·0	0·55	19·0 ± 3·6	< 0·0001	55·4 ± 6·6	0·0032	73·7 ± 4·7	< 0·0001
Low	91·9 ± 2·9		66·3 ± 5·2		32·4 ± 7·0		48·7 ± 9·7		36·0 ± 5·9	
Infused cells ($\times 10^7$/kg)										
< 3·6	83·2 ± 3·7	< 0·0001	51·5 ± 5·0	0·002	23·8 ± 4·8	0·71	49·3 ± 7·3	0·91	57·7 ± 5·4	0·63
≥ 3·6	94·0 ± 2·6		71·4 ± 4·9		27·9 ± 5·5		54·0 ± 9·0		59·5 ± 5·4	
Number of HLA antigen mismatches										
non	92·0 ± 5·4	0·009	64·0 ± 9·6	0·81	26·9 ± 9·5	0·2	69·4 ± 13·3	0·17	48·3 ± 12·6	0·06
I	92·4 ± 2·4		60·2 ± 4·6		28·0 ± 4·6		46·2 ± 6·70		54·4 ± 5·1	
II	73·1 ± 6·9		63·4 ± 7·5		18·9 ± 6·8		54·6 ± 14·2		67·7 ± 7·3	
Acute GVHD										
non-grade I	92·9 ± 3·1	0·53	64·0 ± 5·1	0·15	26·9 ± 5·3	0·39	50·6 ± 7·7	0·93	48·0 ± 5·8	0·77
≥ grade II	89·5 ± 3·1		52·2 ± 6·0		29·6 ± 6·2		54·2 ± 8·9		57·1 ± 7·0	
GVHD prophylaxis										
One drug	88·8 ± 4·4	0·63	72·5 ± 6·2	0·02	5·8 ± 4·8	< 0·0001	70·2 ± 13·2	0·03	73·3 ± 6·7	0·0003
CsA/FK506 + others	90·8 ± 2·8		53·8 ± 4·9		38·7 ± 5·4		44·4 ± 6·9		45·8 ± 5·3	

evaluation, and 20 others were excluded from the evaluation because they developed infections or regimen-related toxicity (RRT) and the final diagnosis of GVHD was not possible. The Kaplan–Meier estimate for developing grade II–IV acute GVHD was 35·2%. Forty-four patients were rated to be grade II, 24 patients were grade III and seven patients were grade IV. According to the univariate analysis, GVHD prophylaxis using a single drug was associated with an increased probability of developing acute GVHD when compared with GVHD prophylaxis using two drugs (51·5% and 33·0% respectively; $P = 0·025$). However, the HLA disparity between donors and recipients and disease status were not significantly associated with an increased probability that the recipient might develop acute GVHD (Table II). The estimated incidence of acute GVHD was 47·1% among the recipients of HLA-identical CB, 29·3% for those given mismatched HLA transplant once and 32·1% for those who received a mismatched transplant twice ($P = 0·25$). From the Cox model, no variables that could be associated with grades II–IV acute GVHD could be found in those patients.

Chronic GVHD affected only 20 of the 115 patients who survived more than 100 d post transplantation with evidence of engraftment.

Relapse

Fifty-five patients (25·4%) relapsed after CBT (23 with acute lymphoblastic leukaemia, 24 with acute myeloid leukaemia, five with MDS, one with CML and two with NHL). Two years after CBT, the probability of relapse was 51·1% (Kaplan–Meier estimate). Details on the univariate analysis for relapse of those with haematological malignancies are shown in Table II. The incidence of relapse was not significantly affected by the frequency of exposure to HLA mismatches (Table II). From the Cox model, patients who underwent transplantation and were at a high risk ($P = 0·04$, RR 1·88, 95% CI 1·03–3·43) had a high probability of relapse.

TRM and causes of death

Ninety-three patients died from transplant-related causes, the Kaplan–Meier estimate for a 1-year TRM being 57·2% of the total population. Details on the univariate analysis for TRM are shown in Table II. The causes of death of overall patients at transplant are reported in Table III. The main causes for CBT-related deaths were infection and respiratory diseases. According to the Cox analysis, patients who underwent transplantation and were at a high risk ($P < 0·0001$, RR 2·80, 95% CI 1·69–4·64) and those who received prophylaxis for acute GVHD using a single drug ($P = 0·0001$, RR 2·45, 95% CI 1·56–3·85) had a higher probability of a 1-year TRM. By Fisher's exact test, proportional deviations between prophylaxis for acute GVHD and the disease status were not found to be significant (data not shown).

Survival

The 3-year probability of OS and EFS was 32·6% and 25·5%, respectively, in the study population (Kaplan–Meier estimate). Details on the univariate analysis for EFS of patients are shown in Table II. The factors influencing EFS were: disease status at CBT, age at CBT and GVHD prophylaxis ($P < 0·0001$, $P = 0·02$ and $P < 0·0001$ respectively; Table II, Figs 1 and 2). According to the Fisher's exact test, proportional deviations between GVHD prophylaxis and disease status, and age and disease category were not significant (data not shown). However, proportional deviations between age at CBT and disease status were significant ($P = 0·01$) using the Fisher's exact test. From the multivariate analysis using Cox's proportional hazards model, it was found that the disease status at CBT and GVHD prophylaxis aggravated the 2-year EFS rate ($P = 0·0013$, RR 1·90, 95% CI 1·28–2·81 and $P = 0·0007$, RR 1·91, 95% CI 1·31–2·79 respectively).

Table III. Cause of death after UCBT.

Causes	
Infection	41
Relapse	18
Respiratory failure	14
Multiorgan failure	8
Veno-occlusive disorder	8
Haemorrhage	6
Renal failure	2
Graft-versus-host disease	2
Thrombotic microangiopathy	2
Unknown	10

DISCUSSION

The most important finding in this investigation was that the GVHD prophylaxis using a single drug was inferior to the two drug regimen for CBT from unrelated donors for haematological malignancies. The incidence of acute GVHD, TRM and the rate of relapse were higher in the group that

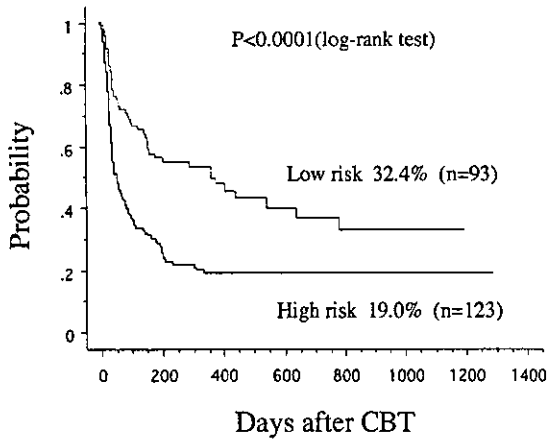

Fig 1. Kaplan–Meier estimate of event-free survival rate according to disease status.

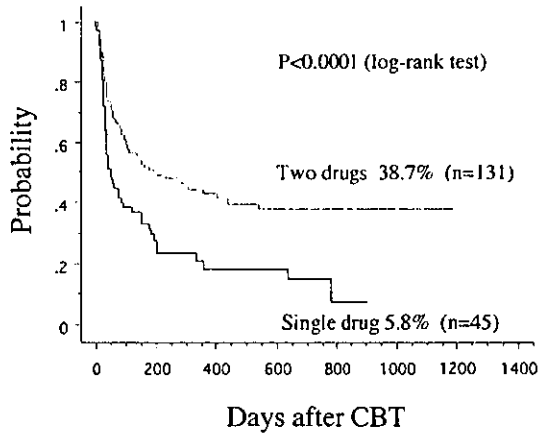

Fig 2. Kaplan–Meier estimate of event-free survival rate according to GVHD prophylaxis.

received a single-drug GVHD prophylaxis. Randomized trials of immunosuppression after HLA-identical BMT showed that the single-agent prophylaxis was associated with a significantly higher incidence of acute GVHD than the combination prophylaxis (Ramsay *et al*, 1982; Storb *et al*, 1986, 1989). Acute GVHD alone or in combination with infection accounts for a significant number of late TRM. As the incidence of GVHD due to HLA incompatibility is less common in CBT, it is possible to treat patients with CBT despite mismatches at two HLA antigen loci. However, the results of the current investigation indicate that the method of immunosuppression for acute GVHD is important even when CBT is selected as the therapeutic approach.

The survival rate was adversely influenced by the TRM in this investigation. As previously reported studies differ in patient disease, race and treatment regimen, it is difficult to compare the outcomes. The slower rate of haematopoietic recovery, the limited number of nucleated cells in the graft and HLA mismatches have been known to be important for the incidence of TRM after CBT from unrelated donors (Gluckman, 2000; Rocha *et al*, 2000). However, the relationship between GVHD prophylaxis and TRM has not been reported. Therefore, a prospective study of GVHD prophylaxis in CBT is desirable. Case series have shown a low incidence of acute and chronic GVHD among the recipients of CBT from unrelated donors, suggesting that these numbers may be lower than those associated with unrelated bone marrow transplantation (UBMT). It should be noted that the vast majority of the unrelated CBT recipients in the present study received an HLA-disparate graft (Barker *et al*, 2001). In UBMT, the HLA disparity has been associated with a very high risk of GVHD (Balduzzi *et al*, 1995). This study supports the contention that an HLA mismatch does not have a major impact on the outcome of CBT from unrelated donors (Wagner *et al*, 1996), as it does in UBMT. Although the reason for this relatively low risk of GVHD in unrelated CBT, despite HLA disparity, has not been elucidated, it may be attributable to a relatively low T-cell dose or the functional immaturity of the neonatal immune system.

Recently, Ohnuma *et al* (2001) reported that HLA-mismatched unrelated CBT is a feasible treatment procedure for a significant proportion of children with leukaemia, especially if conducted during an optimum phase of the disease. Large-scale prospective studies are needed to substantiate this hypothesis.

In July 2001, 4860 CB units were available through the JCBBN for 270 patients who needed HSCT. Suitable donors have been found, with a probability of 1 to every 18 CB units, which is a very high rate when compared with HLA matching at other HSC banks such as the Japan Marrow Donor Programme with a probability of 1–40, and National Marrow Donor Program (USA) with a probability of 1–450. One of the reasons for the higher proportion of suitable donors may be that the donor pool in Japan may be less polymorphic at the HLA locus than other ethnic groups.

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Neuropilin-1 on hematopoietic cells as a source of vascular development

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Neuropilin-1 (NP-1) is a receptor for vascular endothelial growth factor-165 ($VEGF_{165}$) and acts as a coreceptor that enhances the function of $VEGF_{165}$ through VEGF receptor-2 (VEGFR-2). Studies using transgenic and knock-out mice of NP-1 indicated that this molecule is important for vascular development as well as neuronal development. We recently reported that clustered soluble NP-1 phosphorylates VEGFR-2 on endothelial cells with a low dose of $VEGF_{165}$ and rescues the defective vascularity of the NP-$1^{-/-}$ embryo in vitro and in vivo. Here we show that NP-1 is expressed by $CD45^+$ hematopoietic cells in the fetal liver, can bind $VEGF_{165}$, and phosphorylates VEGFR-2 on endothelial cells. $CD45^+NP\text{-}1^+$ cells rescued the defective vasculogenesis and angiogenesis in the NP-$1^{-/-}$ P-Sp (para-aortic splanchnopleural mesodermal region) culture, although $CD45^+NP\text{-}1^-$ cells did not. Moreover, $CD45^+NP\text{-}1^+$ cells together with $VEGF_{165}$ induced angiogenesis in an in vivo Matrigel assay and cornea neovascularization assay. The extracellular domain of NP-1 consists of "a," "b," and "c" domains, and it is known that the "a" and "c" domains are necessary for dimerization of NP-1. We found that both the "a" and "c" domains are essential for such rescue of defective vascularities in the NP-1 mutant. These results suggest that NP-1 enhances vasculogenesis and angiogenesis exogenously and that dimerization of NP-1 is important for enhancing vascular development. In NP-$1^{-/-}$ embryos, vascular sprouting is impaired at the central nervous system (CNS) and pericardium where VEGF is not abundant, indicating that NP-1–expressing cells are required for normal vascular development. (Blood. 2003;101:01801-1809)

Introduction

Blood vessels are formed by 2 different steps called vasculogenesis (formation of new blood vessels de novo) and angiogenesis (branching of pre-existing blood vessels).[1] Over the last several years, a great deal of progress in defining vasculogenesis and angiogenesis has been made by isolating factors including a family of vascular endothelial growth factors (VEGFs), angiopoietins, and ephrins.[2,3] The gene targeting strategy revealed that these factors including counter receptors are important molecules for the development of the vascular system. Among them, VEGFs and cognate receptor tyrosine kinases, tyrosine-kinase VEGF receptor-1 (VEGFR-1) and tyrosine-kinase VEGF receptor-2 (VEGFR-2), which are specifically expressed on the surface of endothelial cells (ECs), have been demonstrated to be required for both vasculogenesis and angiogenesis.[4-7]

The major components of a blood vessel are endothelial cells and mural cells such as smooth muscle cells and pericytes. Therefore, the interaction between endothelial cells and mural cells has been studied in an attempt to elucidate vascular development; however, hematopoietic cells have been suggested to be one of the regulators of the development of blood vessels. Recently, we reported that angiopoietin-1, a ligand for TIE2, is produced by hematopoietic stem cells and that it regulates the formation of the vascular network in the restricted region in vivo.[8] Moreover, VEGF and placental growth factor (PlGF) expressed in erythroblasts have been suggested to be involved in blood vessel formation,[9] and macrophages and mast cells have been reported to be involved in tumor angiogenesis.[10] During embryogenesis and especially organogenesis, tissue-specific cells or cells other than hematopoietic cells may affect vascular development. Therefore, to gain an understanding of the molecular mechanism of vessel formation, it is important to analyze the interactions between various kinds of cells and endothelial cells.

Neuropilin-1 (NP-1) is unique among the many blood vessel–related proteins. NP-1 was initially described as a cell surface glycoprotein expressed on axons in the developing nervous system[11] and was shown to be a receptor for semaphorin/collapsin,[12,13] a family of transmembrane and secreted glycoproteins that act as mediators of neuronal guidance.[12,14] Extracellular domain of NP-1 is composed of complement ("a", CUB), coagulation factor V/VIII ("b"), and MAM ("c") homology subdomains. Secreted collapsin-1 (SemaIIIA) binds to NP-1 on axons, repels neurons, and induces the collapse of neuronal growth cones. Other than the neuronal system, NP-1 was identified as another VEGFR that binds the 165 amino acid form of VEGF ($VEGF_{165}$) but not the 121 amino acid form of VEGF ($VEGF_{121}$).[15] When NP-1 is coexpressed on $VEGFR\text{-}2^+$ ECs, the binding to $VEGF_{165}$ and chemotactic activity by $VEGF_{165}$ for these cells are enhanced compared with those of ECs expressing VEGFR-2 alone; this suggests that in ECs,

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NP-1 acts as a coreceptor for VEGFR-2. Moreover, SemaIIIA inhibits the motility of porcine aortic ECs depending on whether NP-1 is expressed by disrupting the formation of lamellipodia and inducing depolymerization of F-actin.[16] $VEGF_{165}$ and SemaIIIA are competitive inhibitors of each other in binding, in EC motility, and in the collapse assay of dorsal root ganglia,[16] suggesting that the 2 ligands have overlapping NP-1 binding sites, possibly the b/coagulation factor homology domain.[17]

Many studies have recently shown that modification of the *NP-1* gene altered vascular development in vivo. Overexpression of the *NP-1* gene in mice led to the formation of excess capillaries and blood vessels in addition to a malformed heart.[18] Targeted disruption of *NP-1* led to embryonic lethality and vascular defects such as impairment of neural vascularization, transposition of large vessels, and insufficient development of vascular networks in the yolk sac.[19,20] Furthermore, we previously demonstrated that soluble clustered NP-1 enhanced vasculogenesis and angiogenesis in vitro in the $NP\text{-}1^{-/-}$ P-Sp (para-aortic splanchnopleural mesodermal region) culture and in vivo in the $NP\text{-}1^{-/-}$ embryo.[21] Phosphorylation of VEGFR-2 on ECs that had been sorted from an $NP\text{-}1^{-/-}$ embryo was enhanced by simultaneous application of $VEGF_{165}$ and soluble NP-1, compared with that by application of $VEGF_{165}$ alone.[21] It has been demonstrated that stromal cells,[22] tumor cells,[15] and mesenchymal cells[18] express NP-1. These results suggest that the NP-1 on such cells acts on ECs exogenously. We found that $CD45^+$ hematopoietic cells in the murine fetal liver and adult bone marrow express NP-1. In this study, we show that the NP-1 on hematopoietic cells plays a key role in the development of endothelial cells and blood vessel formation.

Materials and methods

Animals

C57BL/6 mice were purchased from Japan SCL (Shizuoka, Japan). NP-1 heterozygous mutant mice[19] and green fluorescence protein (GFP) transgenic mice (gift from Dr Masaru Okabe, Osaka University, Japan)[23] were housed in environmentally controlled rooms of our facility at Kumamoto University School of Medicine under the guidelines of Kumamoto University for animal and recombinant DNA experiments. Genotype analysis of the neuropilin mutants was performed by polymerase chain reaction (PCR) as described by Kitsukawa et al.[19]

RT-PCR analysis

The RNeasy Mini kit (Qiagen, Hilden, Germany) was used for isolation of total RNA from whole embryos, $CD45^+$ cells, or $CD45^-$ cells. Total RNA was reverse transcribed using the reverse transcription (RT) for PCR kit (Clontech, Palo Alto, CA). The cDNA was amplified using Advantage Polymerase Mix (Clontech) in a GeneAmp PCR system model 9700 (Perkin-Elmer, Norwalk, CT) by 30 cycles. The sequences of the gene-specific primers for RT-PCR were as follows: 5′-mNP-1 (ACTGACAGCGCAATAGCAAAAGAAG), 3′-mNP-1 (TCGGACAAATCGAGTATCAGTGGT), 5′-mVEGFR-1 (CTTCCTACAGCACAGCAGATGTGAA), 3′-mVEGFR-1 (CACGTTTACAATGAGAGTGGCAGTG), 5′-mVEGFR-2 (TACACAATTCAGAGCGATGTGTGGT), 3′-mVEGFR-2 (CTGGTTCCTCCAATGGGATATCTTC), 5′-mVEGF165 (CTTTACTGCTGTACCTTCACCATGC), 3′-mVEGF165 (AACAAGGCTCACAGTGATTTTCTGG), 5′-G3PDH (TGAAGGTCGGTGTGAACGGATTTGGC), and 3′-G3PDH (CATGTAGGCCATGAGGTCCACCAC). Each cycle consisted of denaturation at 94°C for 30 seconds and annealing/extension at 70°C for 4 minutes.

Immunohistochemistry

Immunohistochemical analyses on tissue sections and culture dishes were performed as previously described.[24] The tissue fixation procedures were the same as those described by Yoshida et al.[25] The fixed specimens were embedded in polyester wax and sectioned at 8 μm. An anti–PECAM-1 antibody (Pharmingen, San Diego, CA), anti-CD45 antibody (Pharmingen), anti–VEGFR-2 antibody (Pharmingen), anti–vascular endothelial–cadherin (anti–VE-cadherin) antibody (Pharmingen), and anti–NP-1 polyclonal antibody[18] were used in this assay. In brief, anti–PECAM-1 antibody and anti-CD45 antibody were developed with horseradish peroxidase–conjugated antirat immunoglobulin G (IgG) antibody (Biosource, Camarillo, CA), and an anti–NP-1 polyclonal antibody was developed with horseradish peroxidase–conjugated antirabbit IgG antibody (Biosource). In the final step of staining, samples were soaked with phosphate-buffered saline (PBS) containing 250 μg/mL diaminobenzidine (Dojin Chemicals, Kumamoto, Japan) in the presence of 0.05% $NiCl_2$ for 10 minutes, and then hydrogen peroxidase was added for the enzymatic reaction. Finally, the sections were observed and photographed under a microscope (IX-70, Olympus, Tokyo, Japan).

VEGF binding assay and flow cytometry

Fluorokine biotinylated VEGF (R&D Systems, Minneapolis, MN) was used for this binding assay. This assay was performed according to the protocol provided by R&D Systems. In brief, cells from E12.5 fetal liver were stained with a phycoerythrin (PE)–conjugated anti-CD45 monoclonal antibody (mAb) (Pharmingen) and an allophycocyanin (APC)–conjugated anti-B220 mAb (Pharmingen), and then $CD45^+B220^+$ cells were sorted using FACS Vantage (Becton Dickinson, San Jose, CA). A total of 10 μL biotinylated VEGF reagent was added to 25 μL of the $CD45^+B220^+$ cells (4×10^6 cells per milliliter). As a negative control, an identical sample of cells was stained with 10 μL biotinylated negative control reagent. The cells were incubated for 60 minutes on ice. Then, 10 μL avidin–fluorescein isothiocyanate (FITC) reagent was added to each sample, and the reaction mixture was incubated for an additional 30 minutes on ice. After incubation, the cells were washed twice with 2 mL of 1 × RDF1 buffer (wash buffer) to remove unreacted avidin-FITC, and then the cells were resuspended in 0.2 mL of 1 × RDF1 buffer for flow cytometric analysis. The stained cells were analyzed and sorted using FACS Vantage. Total RNA from the sorted $CD45^+B220^+VEGFR^+$ cells was isolated using the RNeasy Mini kit (Qiagen). Then, RT-PCR using various VEGFR-specific primers was performed as described above.

Cell preparation, immunoprecipitation, and immunoblotting

Whole-mount E12.5 embryos were dissociated using Dispase II (Boehringer Mannheim, Mannheim, Germany) and Collagenase S-1 (Nitta Gelatin, Osaka, Japan) and drawn through a 23-gauge needle. Cells were stained with anti–VEGFR-2–PE (Pharmingen) and anti–PECAM-1–biotin (Pharmingen). Biotinylated antibodies were visualized with APC-conjugated streptavidin (Pharmingen). Then, $VEGFR\text{-}2^+PECAM\text{-}1^+$ ECs were sorted using FACS Vantage. Sorted $VEGFR\text{-}2^+PECAM\text{-}1^+$ cells were cultured on OP9 cells in 10% fetal calf serum (FCS) (JRH Bioscience, Lenexa, KS) and 10^{-5} M 2-Mercaptoethanol (2-ME) (GIBCO, Gaithersburg, MD) containing RPMI 1640 (GIBCO) with 5 ng/mL VEGF (PeproTech, London, United Kingdom) and with or without $CD45^+NP\text{-}1^+$ cells and VEGFR-2–Fc fusion protein. After culture for 10 days, cells were stained with an anti–PECAM-1 antibody (Pharmingen) to visualize the existence of ECs. Aggregates consisting of more than 10 cells expressing PECAM-1 were counted as a cluster.

For immunoprecipitation and immunoblotting, sorted $VEGFR\text{-}2^+PECAM\text{-}1^+$ cells were cultured on a fibronectin (FN)–coated dish in 10% FCS supplemented with 10 ng/mL VEGF (PeproTech). After 7 days, culturing cells were washed twice with PBS and incubated in culture in serum-free medium for 12 hour before stimulation. $CD45^+$ cells from E12.5 fetal liver of NP-1 wild and mutant embryos were incubated with VEGF and/or NP-1 Flag, semaphorin IIIA, neutralizing VEGF antibody (Oncogene, Boston, MA) for 30 minutes on ice and then were washed twice with PBS to remove

unreacted VEGF. Then, VEGFR-2$^+$PECAM-1$^+$ ECs were stimulated with CD45$^+$NP-1$^+$ cells or CD45$^+$NP-1$^-$ cells for 10 minutes at 37°C. The cells were solubilized with lysis buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid], pH 7.4; 137 mM NaCl; 5 mM EDTA [ethylenediaminetetraacetic acid]; 1 mM Na_3VO_4, 1% Triton X-100; and protease inhibitors). Immunoprecipitation and immunoblotting were performed as previously described.[26]

Production of recombinant fusion protein

Recombinant fusion proteins of the extracellular domain of murine surface molecules and human IgG (Fc fragment) and Flag epitope were designed. The VEGFR-2–Fc and NP-1 Flag were produced by COS7 cells in serum-free conditioned medium as previously described[21] and purified over a protein A column (Bio-Rad, Richmond, CA) and anti-Flag M2 column (Scientific Imaging System; Eastman Kodak, Rochester, NY). This purity was assessed by Coomassie brilliant blue staining of 7.5% sodium dodecyl sulfate (SDS) gels.

Production of recombinant semaphorin IIIA

Construction of semaphorin IIIA expression vector (pCAG-semIIIA) was previously described.[18] In brief, the full length of semaphorin IIIA cDNA was ligated into the *Eco*RI site of the COS cell expression vector pCAGGS. COS cells were transfected with pCAG-semIIIA using LipofectAMINE (GIBCO) and cultured in Dulbecco modified Eagle medium (DMEM) containing 5% FCS (JRH Bioscience) for 4 days at 37°C. Then culture supernatant was collected.

In vitro culture of P-Sp

The stromal cell line, OP9,[27] was maintained in α-modified minimum essential media (α-MEM) (GIBCO) supplemented with 20% FCS (JRH Bioscience). Explants of E9.5 P-Sp containing a part of the omphalomesenteric artery (OA) were cultured on OP9 stromal cells in 10% FCS and 10^{-5}M (2-ME) (GIBCO) containing RPMI 1640 (GIBCO). After culture for 14 days, an anti–PECAM-1 antibody (Pharmingen) was used to visualize the existence of ECs.

Quantitative analysis of vascular areas

The method of immunohistochemistry in the P-Sp culture using PECAM-1 antibodies was performed as described above. After PECAM-1 immunohistochemical staining, images were integrated using a color chilled 3CCD camera (Hamamatsu Photonics, Shizuoka, Japan). Image processing software (NIH image 1.62/Power Macintosh G3; National Institutes of Health, Bethesda, MD) was used to determine alterations in the size of vascular areas. Three vascular areas from each P-Sp explant were measured under each culture condition. The values of all parameters are shown as the mean and standard deviation. *P* values were calculated by 2-tailed Student *t* test analysis.

In vivo neovascularization using Matrigel

Preparation, injection, and processing of Matrigel (Becton Dickinson) were performed as described by Passaniti et al[28] with some modifications. Briefly, 8-week-old C57BL mice were injected subcutaneously with 0.5 mL Matrigel and 40 units of heparin per milliliter (Sigma), 20 ng/mL VEGF (PeproTech), and 5×10^4 CD45$^+$B220$^+$ cells from the fetal liver of E12.5 NP-1 wild or mutant embryos. Five days later, the mice were killed and Matrigel plugs were removed, weighed, and processed for histology or hemoglobin concentration determination. For histologic analysis, plugs were fixed by 4% paraformaldehyde and embedded in polyester wax and sectioned at 8 μm. Then sections were stained with anti–PECAM-1 antibody (Pharmingen) or anti–VEGFR-2 antibody (Pharmingen). For hemoglobin determination, which correlates with the number of blood vessels, plugs were homogenized in 1 mL distilled water. Hemoglobin concentration was determined by the Drabkin method using a Drabkin reagent kit (Sigma).

Mouse cornea neovascularization assay

The corneal assay was performed as previously described.[29,30] In brief, under sterile conditions, slow-release pellets were prepared incorporating VEGF (PeproTech) alone, PBS alone, VEGF$^+$CD45$^+$NP-1$^+$ cells, and VEGF$^+$CD45$^+$NP-1$^-$ cells into a casting solution of ethynil-vinyl copolymer (Elvax-40, DuPont, Wilmington, DE) in 10% methylene chloride. After anesthesia with sodium pentobarbital (Dainippon Pharmaceutical, Osaka, Japan), pellets were implanted into the corneal micropocket in male 8-week-old C57BL/6 mice. The corneas of all mice were routinely examined by slitlamp biomicroscopy on postoperative days 5 or 6 after pellet implantation. After taking photographs, organs were excised, embedded in polyester wax, and sectioned at 10 μm. Staining of sections was performed as described above using anti–PECAM-1 antibody (Pharmingen), anti–VEGFR-2 antibody (Pharmingen), or anti–VE-cadherin antibody (Pharmingen).

Expression of mutant neuropilin-1 proteins in L cells

L cells transfected with the cDNA of various mouse NP-1 mutants were produced as described previously.[31] In brief, the myc-tag sequence, GGEQKLISEEDL, was introduced in such cDNA as follows. An *Xba*I site was added to the 3′-end of the coding region of NP-1 by PCR, and then the *Xba*I-myc-tag-stop codon adapter was ligated. In all mutant NP-1 cDNAs, the signal sequence was retained intact. To isolate cells that stably expressed truncated NP-1, L cells, a mouse fibroblastic cell line, were cotransfected with the truncated NP-1 cDNAs and pST-neoB[32] according to the calcium phosphate method and selected with Geneticin (GIBCO).

Results

NP-1 expression on hematopoietic cells

To determine whether hematopoietic cells (HCs) express NP-1, several fractions of CD45$^+$ cells from murine fetal liver at E12.5 were sorted, and the expression of NP-1 and $VEGF_{165}$ mRNA was examined by RT-PCR. In the fetal liver, high NP-1 expression was detected in CD45$^+$B220$^+$ B lymphocytes, CD45$^+$Mac-1$^+$ monocytes, and CD45$^-$Ter119$^+$ erythrocytes. In contrast, NP-1 was less expressed in CD45$^+$CD4$^+$/CD8$^+$ T lymphocytes and CD45$^+$CD34$^+$c-kit$^+$ stem cells (Figure 1A). In the case of $VEGF_{165}$ expression, high $VEGF_{165}$ expression was detected in CD45$^+$Mac-1$^+$ monocytes and CD45$^-$Ter119$^+$ erythrocytes as previously reported.[9] Next, we investigated whether nonendothelial cells expressing NP-1 were located at the site of vascular sprouting, focusing on the central nervous system (CNS) where vascular defects had been observed in NP-1$^{-/-}$ embryos. In the CNS, round cells in the lumen expressed NP-1 (Figure 1Bii; arrows), and these round cells were in the hematopoietic lineage as confirmed by their expression of CD45 in the serial sections (Figure 1Biv; arrows). ECs (arrowheads in Figure 1Bii) and neuronal cells in dorsal root ganglia (DRG; Figure 1Bi,iii) were also NP-1$^+$ as previously reported.[19,21]

NP-1 on hematopoietic cells binds $VEGF_{165}$ and induces phosphorylation of VEGFR-2 on ECs

To determine whether NP-1 on CD45$^+$ HCs binds $VEGF_{165}$, we performed flow cytometric analysis. As shown in Figure 1, a portion of the CD45$^+$ HCs expressed NP-1. It was previously reported that monocytes such as Mac-1$^+$ cells express VEGFR-1.[33,34] According to our analysis, CD45$^+$B220$^+$ HCs did not express VEGFR-1 or VEGFR-2 (data not shown). Therefore, in the binding assay for $VEGF_{165}$, we used CD45$^+$B220$^+$ cells so that we could study the binding of $VEGF_{165}$ to NP-1 other than VEGFR-1 and VEGFR-2. First, we obtained CD45$^+$B220$^+$ cells

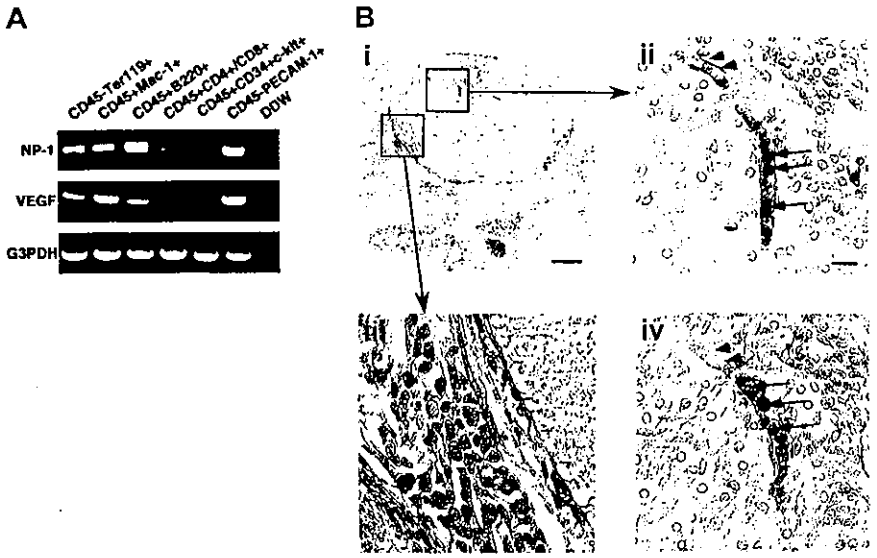

Figure 1. NP-1 expression on hematopoietic cells. (A) RT-PCR analysis of NP-1 and VEGF-A expression in various fractions of hematopoietic cells from the fetal liver. High NP-1 expression was detected in $CD45^+B220^+$ and $CD45^+Mac\text{-}1^+$ cells; however, very low NP-1 expression was detected in the fraction of stem cells marked as $CD45^+CD34^+c\text{-}kit^+$ cells. RNA from CD45–PECAM-1^+ ECs was used for positive control. (B) Histologic analysis of NP-1 expression on hematopoietic cells in embryo. (i) Transversal section of spinal cord stained with anti–NP-1 antibody. Boxes are viewed in higher power in panels ii and iii. (ii) Round cells in the lumen of a vessel (arrows) and ECs lining the vessel (arrowheads) are positively stained with anti–NP-1 antibody. (iii) Neuronal cells in the DRG are positively stained with anti–NP-1 antibody. (iv) Serial section of panel ii stained with anti-CD45 antibody. Round cells in the vessel as observed in panel ii are positively stained with anti-CD45 antibody. Positively stained cells are visualized as dark blue products. Isotype-matched control Ig for anti-CD45 or anti–NP-1 antibody did not show any staining. Scale bar indicates 50 μm (i,iii); 20 μm (ii,iv).

(2.6%, Figure 2Ai) from fetal livers and incubated the cells with biotinylated $VEGF_{165}$. The binding of $VEGF_{165}$ to cells was visualized by staining with streptavidin-FITC (Figure 2Ai). Interestingly, most $CD45^+B220^+$ cells were bound to $VEGF_{165}$. We isolated these $VEGF_{165}$ receptor-positive cells (approximately 50% of VEGF-binding cells, as shown in Figure 2Ai), and the mRNA from these cells was analyzed for the expression of VEGF receptors by RT-PCR. As expected, $CD45^+B220^+$ cells expressed NP-1 (Figure 2Aii) but did not express other $VEGF_{165}$ receptors. Therefore, we concluded that $CD45^+B220^+$ B lymphocytes express NP-1, which binds with $VEGF_{165}$.

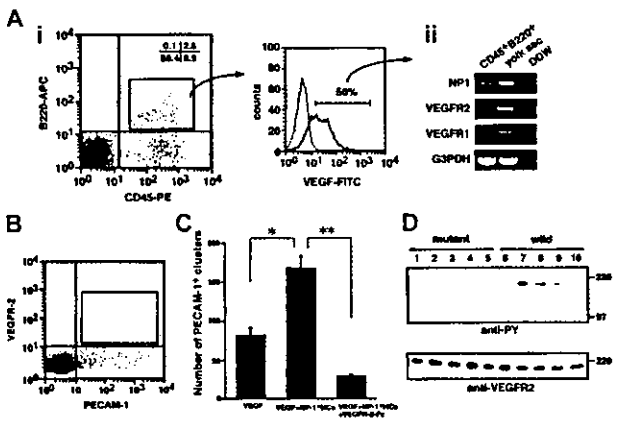

Figure 2. $CD45^+$ cells isolated from the fetal liver bind $VEGF_{165}$ and phosphorylate VEGFR-2 on ECs. (A) Cells obtained from an E12.5 fetal liver were stained with anti-CD45–PE and anti-B220–APC and analyzed by flow cytometry. Some cells (2.6%) were positively stained by both antibodies (i), and sorted $CD45^+B220^+$ cells were then stained with VEGF-biotin and streptavidin-FITC. Many of the cells shifted to the right (blue line; positive cells). Fifty percent (50%) of the cells over the negative gate (i; right histogram) expressed NP-1 alone among $VEGF_{165}$ receptors (ii). (B) E12.5 embryos were dissociated and stained with PE-conjugated anti–PECAM-1 and biotin-conjugated anti–VEGFR-2 mAbs. Biotin was developed to avidin-allophycocyanin. PECAM-1^+VEGFR-2^+ cells indicated by the box were sorted by using FACS Vantage. (C) PECAM-1^+VEGFR-2^+ cells were cultured with OP9 cells in the presence or absence of VEGF, NP-1^+ HCs, and VEGFR-2–Fc. The number of PECAM-1^+ endothelial clusters was scored. *P, **$P < .05$. (D) Cell lysates of PECAM-1^+VEGFR-2^+ cells that had been stimulated by various factors or cells were immunoprecipitated with anti–VEGFR-2 antibody and then subjected to Western blotting using an antiphosphotyrosine mAb (anti-PY). The PECAM-1^+VEGFR-2^+ cells were incubated with the $CD45^+$ cells from fetal livers of NP-1 mutant (lanes 1-5) or wild-type (lanes 6-10) embryos with or without the indicated factors in each lane; no factor (lanes 1, 6); 10 ng/mL VEGF (lanes 2, 7); 10 ng/mL VEGF plus 20 μg/mL NP-1 Flag (lanes 3, 8); 10 ng/mL VEGF plus 300 ng/mL SemaIIIA (lanes 4, 9); 10 ng/mL VEGF plus 1 μg/mL anti-VEGF neutralizing antibody (lanes 5, 10). Phosphorylation of VEGFR-2 was induced by the addition of $CD45^+NP1^+$ cells mixed with 10 ng/mL VEGF (lane 7), and it was specifically blocked by NP-1 Flag or SemaIIIA or anti-VEGF neutralizing antibody (lanes 8-10). The addition of $CD45^+NP1^-$ cells mixed with VEGF barely induced phosphorylation of VEGFR-2 on ECs (lane 2). "wild" indicates cells from the wild type, and "mutant" indicates cells from the mutant embryo of NP-1 from the same litter.

To examine whether NP-1 on $CD45^+$ HCs directly stimulates VEGFR-2 on ECs, we sorted VEGFR-2^+PECAM-1^+ ECs from E12.5 embryos (Figure 2B) and cultured them on OP9 cells in the presence or absence of VEGF, NP-1^+ HCs, and VEGFR-2–Fc. After 10 days of culturing, cultured cells were fixed and stained with anti–PECAM-1 antibody to examine the proliferation of ECs. VEGFR-2^+PECAM-1^+ ECs from embryos formed vascular structure (data not shown) in the presence of 5 ng/mL VEGF. On addition of $CD45^+$ HCs from fetal liver of wild-type embryos to this culture, the number of PECAM-1^+ EC clusters significantly increased (Figure 2C). This effect was completely abolished by addition of VEGFR-2–Fc (Figure 2C). $CD45^+$ cells from NP1 mutant embryos did not enhance the proliferation of ECs in the presence of 5 ng/mL VEGF (data not shown). These findings indicate that $CD45^+NP\text{-}1^+$ HCs enhance the proliferation of individual ECs through VEGFR-2.

To examine the role of NP-1 on $CD45^+$ in the activation of VEGFR-2, we studied the phosphorylation of VEGFR-2 on ECs. For this experiment, we sorted VEGFR-2^+PECAM-1^+ ECs from E12.5 embryos (Figure 2B) and cultured on a fibronectin-coated dish. After 7 days of culturing, ECs were starved under serum-free condition for 12 hours and subsequently challenged with $CD45^+$ hematopoietic cells mixed with $VEGF_{165}$ in the presence or absence of an NP-1 Flag or semaphorin IIIA or anti-VEGF neutralizing antibody. Cell lysates were immunoprecipitated with an anti–VEGFR-2 antibody and then subjected to Western blotting using an antiphosphotyrosine mAb (4G10). Phosphorylation of VEGFR-2 was induced by adding $CD45^+NP1^+$ cells mixed with $VEGF_{165}$, and it was specifically blocked by the NP-1 Flag or semaphorin IIIA or neutralizing VEGF antibody, although the addition of $CD45^+NP1^-$ cells mixed with the same amount of VEGF as above did not induce phosphorylation of VEGFR-2 on ECs (Figure 2D). We confirmed that the mRNA expression of angiogenic factors such as VEGFs and angiopoietin-1 and -2 did not differ between the $CD45^+NP1^+$ cells and $CD45^+NP1^-$ cells (data not shown).

Therefore, we concluded that NP-1 on hematopoietic cells together with $VEGF_{165}$ activates VEGFR-2 on ECs exogenously.

Hematopoietic cells expressing NP-1 induce vascular development in in vitro P-Sp culture

We previously reported that soluble clustered NP-1 enhanced vascular development.[21] Because some $CD45^+$ cells expressed NP-1, we hypothesized that $CD45^+$ cells work as soluble NP-1 receptors, because hematopoietic cells can freely move through the vessels. To address this hypothesis, we used the P-Sp culture system.[24] As previously reported, this P-Sp culture system supports the simultaneous growth of ECs and hematopoietic cells.[24] Especially in the case of ECs, the process of sheet formation occurs first (vascular bed), and subsequently the spreading ECs form a network (vascular network). We found that inhibition of VEGF signaling in this culture system using soluble Flk-1/VEGFR-2 led to complete lack of EC development,[24] inhibition of angiopoietin signaling in this culture system using soluble TIE2 led to suppress the network formation,[24] and addition of an excess amount of VEGF in the culture enhanced sheet formation.[21] In the case of hematopoietic cells, the hematopoietic stem cells that develop first on the sheet formation of ECs migrate into the network area of ECs and proliferate and differentiate on the network area.[24] Recently, we reported the critical role of angiopoietin-1 produced from hematopoietic stem cells in promoting the formation of this network.[8] Therefore, if the NP-1 on hematopoietic cells works like exogenously added soluble NP-1, such hematopoietic cells should rescue the defective vascularity observed in the P-Sp culture of $NP\text{-}1^{-/-}$ embryos. Therefore, we tested whether $CD45^+$ cells expressing NP-1 could rescue the defective vascular formation in the culture of $NP\text{-}1^{-/-}$ P-Sp explants.

In $NP\text{-}1^{-/-}$ embryos, formation of both the vascular bed and network was defective (Figure 3Ai). The addition of $CD45^+NP\text{-}1^+$ cells from the fetal liver of E12.5 green mice expressing GFP ubiquitously (5×10^3 cells per well) rescued the defective formation of the vascular bed and network (Figure 3Aii). The exogenously added hematopoietic cells did not differentiate to endothelial cells as confirmed by their morphology (Figure 3Aiv). On the other hand, the addition of $CD45^+$ cells from the fetal liver of E12.5 $NP\text{-}1^{-/-}$ embryos did not effectively rescue the defective vascular formation (Figure 3Aiii). Quantitative analyses show that vascular areas were increased in the presence of $CD45^+NP\text{-}1^+$ cells (Figure 3B).

Hematopoietic cells expressing NP-1 induce angiogenesis in in vivo Matrigel assay

To confirm whether $CD45^+$ hematopoietic cells induce angiogenesis in vivo, we used the Matrigel assay system[28] and cornea neovascularization assay system,[29,30] which have been established as a method for evaluating angiogenesis in vivo.[28] In Matrigel assay, appearance of Matrigel containing $CD45^+B220^+$ cells from the E12.5 fetal liver of wild-type mice together with $VEGF_{165}$ was yellow (Figure 4Aii). In the section, $PECAM\text{-}1^+$ (Figure 4Aiv) or $VEGFR\text{-}2^+$ (Figure 4Avi) ECs formed blood vessels in the Matrigel. On the other hand, appearance of Matrigel containing $CD45^+B220^+$ cells from NP1 mutants together with $VEGF_{165}$ was white (Figure 4Ai). In the section, we did not detect $PECAM\text{-}1^+$ (Figure 4Aiii) or $VEGFR\text{-}2^+$ (Figure 4Av) ECs. Figure 4B shows the hemoglobin content normalized to the weight of the Matrigel plugs. As previously reported,[28] the hemoglobin content correlates with the number of blood vessels in the plugs. By this method, $CD45^+NP\text{-}1^+$ cells with $VEGF_{165}$ induced significantly more blood vessels in the Matrigel plugs compared with $CD45^+NP\text{-}1^-$ cells with $VEGF_{165}$.

Hematopoietic cells expressing NP-1 induce angiogenesis in corneal neovascularization assay

For further confirmation of the effect of $CD45^+NP\text{-}1^+$ HCs in vivo for vascular formation, we used corneal neovascularization assay. In this assay (Figure 5), $CD45^+$ cells from the E12.5 fetal liver of wild-type embryos together with a low dose of VEGF (20 ng/mL) induced neovascularization (Figure 5Aiii) as well as a high dose of VEGF (100 ng/mL) as a positive control (Figure 5Aiv). On the other hand, pellet alone or $CD45^+$ cells from the E12.5 fetal liver of NP-1 mutants together with a low dose of VEGF (20 ng/mL) did not induce neovascularization (Figure 5Ai-ii). In the section, we confirmed that pellet containing a low dose of VEGF and $CD45^+$ cells from wild-type embryos induced blood vessels composed of $PECAM\text{-}1^+$ (Figure 5Bii), $VEGFR\text{-}2^+$ (Figure 5Biv), or VE-cadherin$^+$ (Figure 5Bvi) ECs in cornea and that pellet containing PBS alone did not (Figure 5Bi,iii,v).

In these 2 types of in vivo analyses, induction of angiogenesis by $CD45^+$ cells with a low dose of VEGF was almost completely

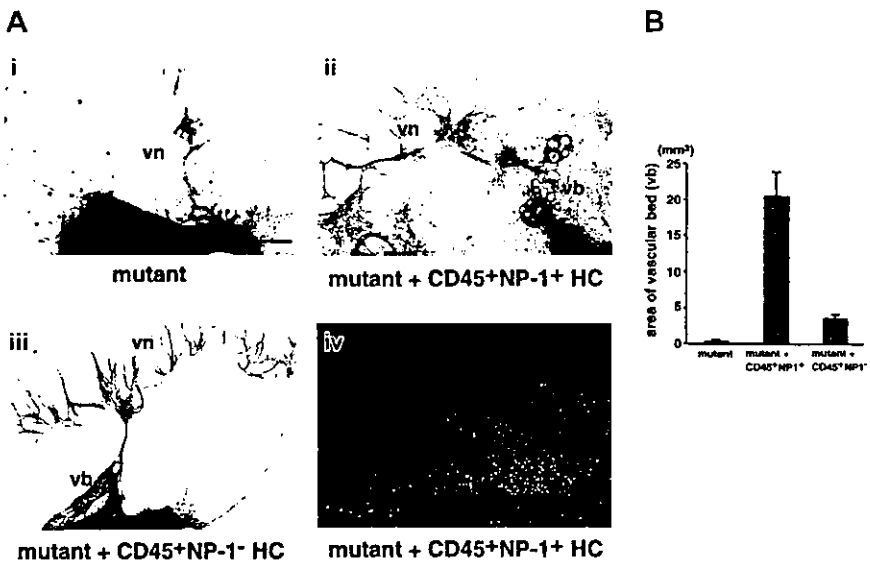

Figure 3. Effect of hematopoietic cells expressing NP-1 on vascular development in the P-Sp culture. (A) The development of ECs in P-Sp cultures from E9.5 $NP\text{-}1^{-/-}$ embryos. Culture plates were fixed after 14 days of culture and stained with anti–PECAM-1 mAb. $PECAM\text{-}1^+$ cells are visualized as dark blue products. (i) In mutant embryo, formation of the vascular bed and network is defective. (ii) The addition of $CD45^+$ cells from the fetal liver of E12.5 GFP embryo (5×10^3 cells per well), which are also positive for NP-1, rescued the defective formation of the vascular bed (vb) and network (vn); however, these cells did not differentiate to endothelial cells as confirmed by their morphology (iv). (iii) The addition of $CD45^+$ cells from the fetal liver of E12.5 $NP\text{-}1^{-/-}$ embryo did not rescue the defective vascular formation. (iv) Localization of GFP^+ HCs in panel ii. Formation of vb and vn observed in panel ii is comparable to that observed in the culture using P-Sp explants from wild-type embryo. Scale bar indicates 200 μm. (B) Comparison of the area of the vascular bed. The area of the vascular beds in the images in panel A was determined by NIH image 1.62 software. The vascular area per explant is as follows: (i) 0.3 ± 0.1 mm²; (ii) 20.5 ± 3.2 mm²; (iii) 3.5 ± 0.5 mm². Each result was obtained from 3 independent experiments and is expressed as the mean ± SD.

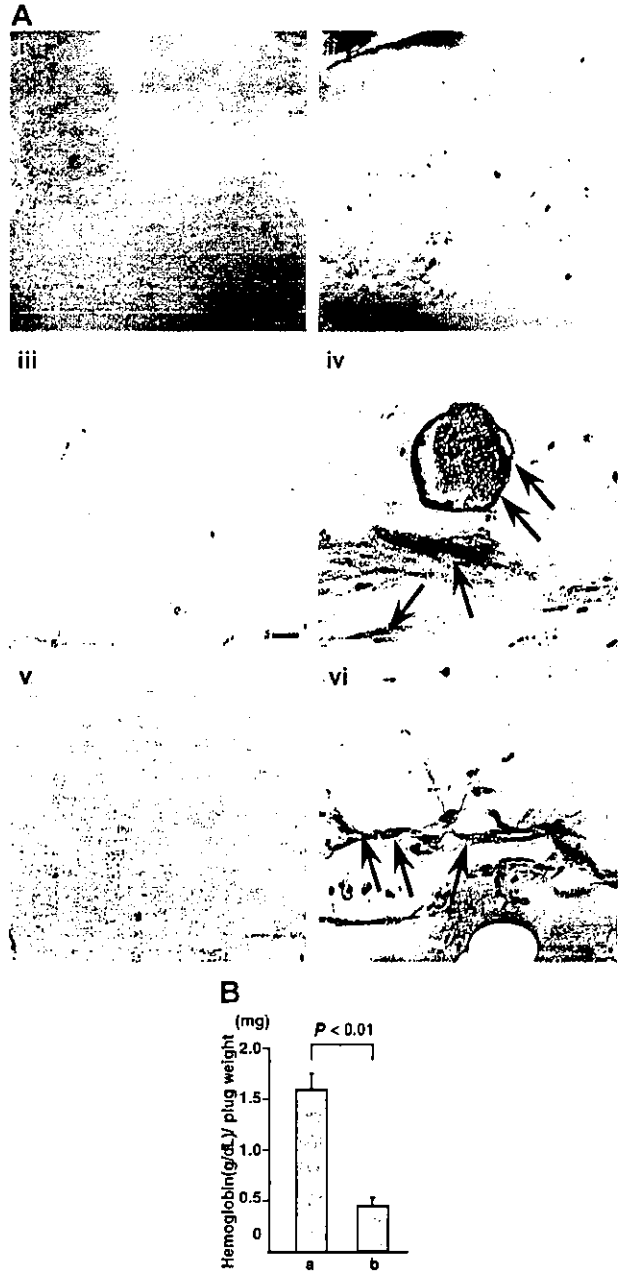

Figure 4. Hematopoietic cells expressing NP-1 induce angiogenesis in in vivo Matrigel assay. (A) Matrigels containing $CD45^+B220^+$ cells from E12.5 fetal liver of wild type (ii,iv,vi) or $CD45^+B220^+$ cells from E12.5 fetal liver of NP-1 mutants (i,iii,v) were injected subcutaneously near the abdominal midline of 8-week-old C57BL mice. (i-ii) Gross appearance of Matrigels on day 5. (iii-vi) Histologic analysis of sections from the Matrigels. ECs were visualized by the staining with anti–PECAM-1 antibody (iii-iv) or anti–VEGFR-2 antibody (v,vi). Scale bar indicates 500 μm (i-ii) and 50 μm (iii-vi). Arrows indicate newly formed vessels in the Matrigel plugs. (B) Values represent the concentration of hemoglobin (g/dL) per Matrigel plug weight (mg) ± SE for 6 assays. "a" is $CD45^+$ NP-1^+ cells with $VEGF_{165}$; "b," $CD45^+$ NP-1^- cells with $VEGF_{165}$.

suppressed by simultaneous addition of soluble NP-1 Flag, SemaIIIA, or neutralizing antibody against VEGF (data not shown).

Dimerization of neuropilin-1 is important for inducing angiogenesis

Previously we reported that the dimer of soluble NP-1 induces the phosphorylation of VEGFR-2 and enhanced vascular development, although the monomer of soluble NP-1 did not.[21] It is known that the "a" and "c" domains of the NP-1 protein are required for dimerization of NP-1.[31] We previously constructed L cells that expressed various portions of the extracellular domain of NP-1.[31] Upon the addition of L cells that possessed only the "a" domain (data not shown) or "a" and "b" domains of the NP-1 protein (Figure 6A-C) to the NP-$1^{-/-}$ P-Sp culture (2×10^3 cells per well) after 4 days of starting culture, the defective vascularity was not rescued at either the areas adherent to (Figure 6B) or the areas nonadherent to the L cells (Figure 6C). On the other hand, upon the addition of L cells that possessed the "a," "b," and "c" domains (Figure 6D-F) to the NP-$1^{-/-}$ P-Sp culture (2×10^3 cells per well) at the same schedule as above, the defective vascularity was rescued at the area adherent to the L cells (Figure 6E); however, it was not rescued at the areas that were nonadherent to the L cells (Figure 6F).

These results indicated that dimerization of NP-1 on the cell surface is important for inducing angiogenesis at the site where NP-1^+ cells and ECs colocalize.

Discussion

In this report, we found that the NP-1 expressed on hematopoietic cells regulates vascular development by exogenously affecting the stimulation of VEGFR-2. Moreover, we found that this kind of exogenous effect was exerted not only by hematopoietic cells but also by stromal cells (data not shown) and L cells (fibroblastic cell lines). Previously we reported that the addition of the soluble form of clustered NP-1 with a low dose of $VEGF_{165}$ effectively induced phosphorylation of VEGFR-2 compared with the addition of a low dose of $VEGF_{165}$ alone and that the defective vasculature of NP-1 mutant mice was rescued by injection of soluble clustered NP-1 in utero.[21] Taking together our present report and previous findings, we confirmed that NP-1 on hematopoietic cells other than endothelial cells can induce vascular development by working as a soluble clustered NP-1 (Figure 7).

Previously, we reported the biologic differences between the monomer and dimer types of soluble NP-1.[21] Monomer-type NP-1 bound and saturated $VEGF_{165}$ and inhibited VEGF-VEGFR signaling. On the other hand, dimer-type NP-1 enhanced $VEGF_{165}$-mediated VEGFR-2 phosphorylation. This biologic effect corresponds to that of the physiological soluble NP-1. Naturally occurring soluble NP-1 contains only the "a" and "b" domains of NP-1 and lacks the "c" domain.[35] Because the "a" and "c" domains of NP-1 have been suggested to be important for dimerization,[31] such natural soluble NP-1 exists as a monomer form. In this case, such spliced NP-1 has been suggested to inhibit tumor angiogenesis by saturating VEGF.[36] Our analyses with the in vivo Matrigel and cornea neovascularization assay as well as the in vitro P-Sp culture clearly showed that exogenous NP-1 on hematopoietic cells enhanced vascular development. However, it was unclear whether NP-1 on hematopoietic cells had to undergo dimerization to enhance vascular development. Therefore, we generated several sublines of L cells harboring mutated NP-1, such as NP-1 with the "a" domain alone, NP-1 with the "a" and "b" domains, and NP-1 with the "a", "b", and "c" domains, and then added the cells into a P-Sp culture of an NP-1 mutant. The results clearly showed that only the dimer form of exogenous NP-1 on the cell surface could rescue the defective vasculature of the NP-1 mutant. Although it is not clarified whether NP-1 on hematopoietic cells is always undergoing dimerization, we may conclude that at least the dimer form of NP-1 on the cell surface can exogenously stimulate VEGFR-2 on endothelial cells. Moreover, we could not deny the possibility that hematopoietic cells release the dimer form of soluble NP-1 by proteolytic cleavage. However, we could not detect soluble NP-1 in the culture supernatant of hematopoietic