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Up-Regulation of Vascular Endothelial Growth Factor (VEGF) in Small-for-Size Liver Grafts Enhances Macrophage Activities through VEGF Receptor 2-Dependent Pathway[1]

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This study aims to investigate the potential role of vascular endothelial growth factor (VEGF) and VEGF-R2 (fetal liver kinase (Flk)-1) in mediating macrophage activities in small-for-size liver transplantation. A rat orthotopic liver transplantation model was performed using either whole, 50, or 30% liver grafts (both 50 and 30% were regarded as small-for-size) in syngeneic or allogeneic combinations, respectively. Firstly, the mRNA and protein levels of VEGF and Flk-1 in liver grafts were detected by RT-PCR and Western blot, and the number of Flk-1$^+$ macrophages (labeled by ED1) was determined by flow cytometry. It was found that the small-for-size isografts and allografts presented higher levels of VEGF and Flk-1 expression than the whole isograft and allograft. In addition, a higher number of Flk-1$^+$ED1$^+$ cells were detected in the small-for-size isografts and allografts than the whole isograft and allograft. Secondly, our study revealed that macrophage cell lines did not initially express detectable Flk-1, but could be induced by VEGF, and the inducible expression of Flk-1 in macrophages was related to their migration and proliferation activities. Finally, our study demonstrated that the induction of Flk-1 expression on macrophages by VEGF was associated with the expression of NF-κB and heat shock protein 90. In conclusion, the present study showed that the up-regulated expression of VEGF and its interaction with Flk-1 in small-for-size liver grafts might facilitate the activities of macrophages. ***The Journal of Immunology*****, 2004, 173: 2507–2515.**

Partial liver transplantation is a valuable strategy to solve the problem of organ shortage. However, small-for-size liver grafts encounter several risks of graft failure after reperfusion, including microcirculatory damage (1), more severe inflammatory responses (2), and accelerated acute rejection process (3). It was hypothesized that the accelerated acute rejection process was related to liver regeneration, based on the observation that alloantigen presentation was augmented in these grafts (4). In addition, our previous study revealed that early activation of macrophages in small-for-size allografts might contribute to the accelerated immune attacks (5). However, the mechanism that leads to early activation of those macrophages is not clear.

Similar to a liver remnant after partial hepatectomy, the transplanted small-for-size liver grafts also undergo regeneration after reperfusion, during which angiogenesis plays an essential role. Vascular endothelial growth factor (VEGF)[3] secreted by replicating hepatocytes is a key player in the process of angiogenesis (6, 7), in which its major effects are on endothelial cell division and migration (8, 9). However, VEGF is also found to be a chemotaxic mediator of human monocytes (10), and a recent study even revealed that VEGF possessed proinflammatory properties that attracted leukocyte activation (11). Some other studies demonstrated that VEGF could enhance cardiac allograft arteriosclerosis (12), and might be used as a surveillance marker for cellular rejection in cardiac transplantation (13). Importantly, the effects of VEGF on its target cells are closely related to its interaction with VEGF receptors, among which VEGF-R2 (fetal liver kinase (Flk)-1) mediates most of the biological functions of VEGF. However, whether VEGF and Flk-1 in the regenerating small-for-size liver grafts, especially in the allogeneic setting, participates in mediating inflammatory and acute rejection responses remains to be determined. Therefore, in this study, we tried to elucidate the expression of VEGF and Flk-1 in liver grafts, and explored the potential role of VEGF-Flk-1 interaction in mediating the activities of macrophages.

Materials and Methods

Animal and experimental groups

Orthotopic liver transplantation (14) was performed using adult male Dark Agouti (RT1^a) and Lewis (RT1^l) rats as donors and recipients, respectively. The percentage of grafts was determined according to experimental protocols. A total of six experimental groups were included: 1) whole isograft (Lewis to Lewis), $n = 9$; 2) 50% isograft, $n = 9$; 3) 30% isograft, $n = 9$; 4) whole allograft (Dark Agouti to Lewis), $n = 12$; 5) 50% allograft, $n = 12$; and 6) 30% allograft, $n = 12$. Both 50 and 30% grafts were regarded as small-for-size grafts. No treatment was given to the groups. Animals were sacrificed at 6, 24, and 72 h after reperfusion (3–4 animals at each time point in each group) for tissue and plasma collection.

RT-PCR, Western blot, and ELISA

Total RNA extraction, first-strand DNA synthesis, and PCR were performed according to the methods previously described (5). Primers for

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[3] Abbreviations used in this paper: VEGF, vascular endothelial growth factor; Hsp90, heat shock protein 90; Flk, fetal liver kinase; SU1498, (E)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)amino-carbonyl]acrylonitrile.

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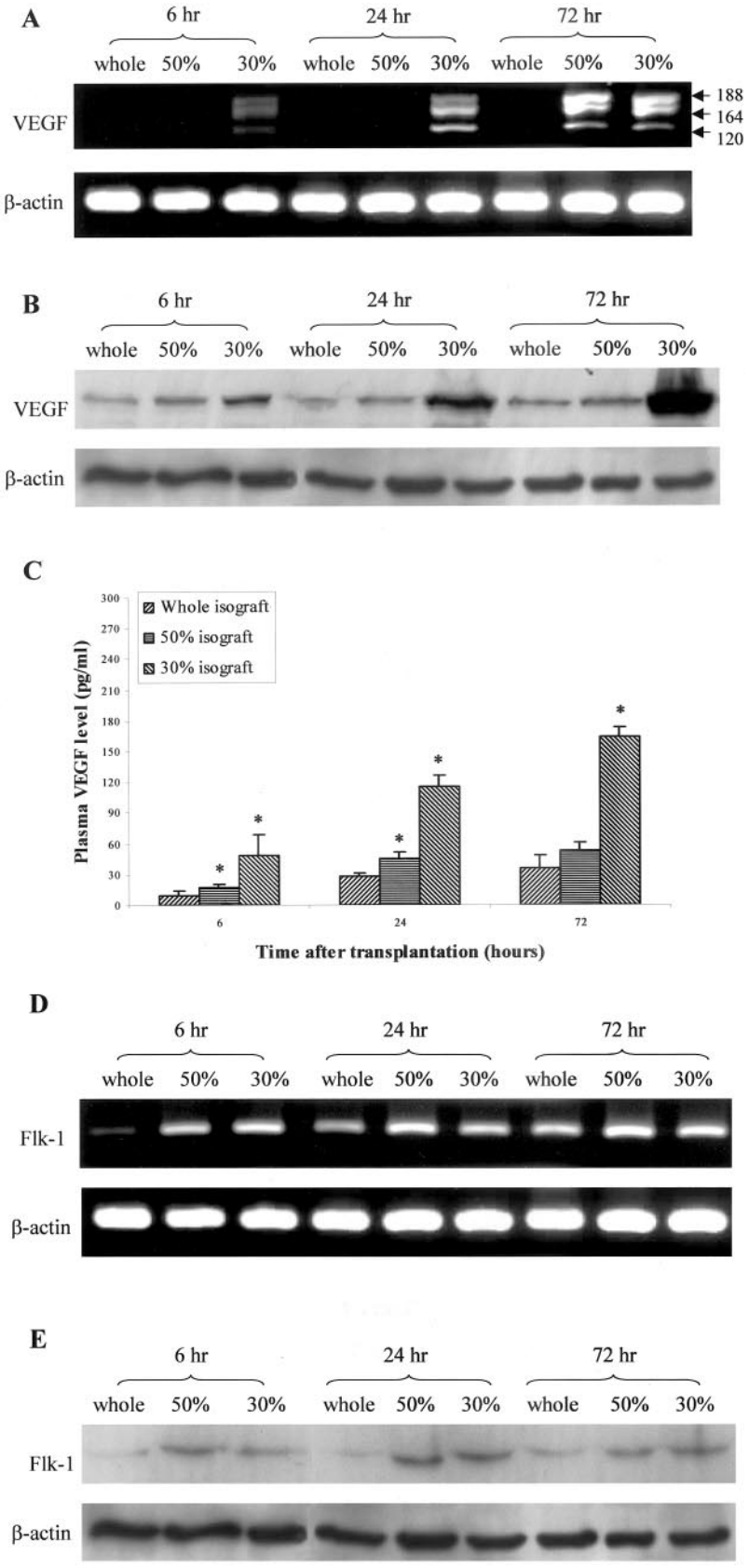

FIGURE 1. VEGF and VEGF-R2 (Flk-1) expression in isografts during early phase after reperfusion. Higher levels of VEGF mRNA (*A*) and protein (*B*) were observed in the 30% isograft than that in the whole isograft, starting from 6 h after reperfusion. The three bands in *A* represented VEGF isoforms 120, 164, and 188. The soluble VEGF (*C*) in plasma displayed a similar pattern as in the isografts. The expression of Flk-1 at both mRNA (*D*) and protein (*E*) levels was also higher in the 50 and 30% isograft groups. $*$, $p < 0.05$, compared with whole isograft group. There were three animals at each time point in each group.

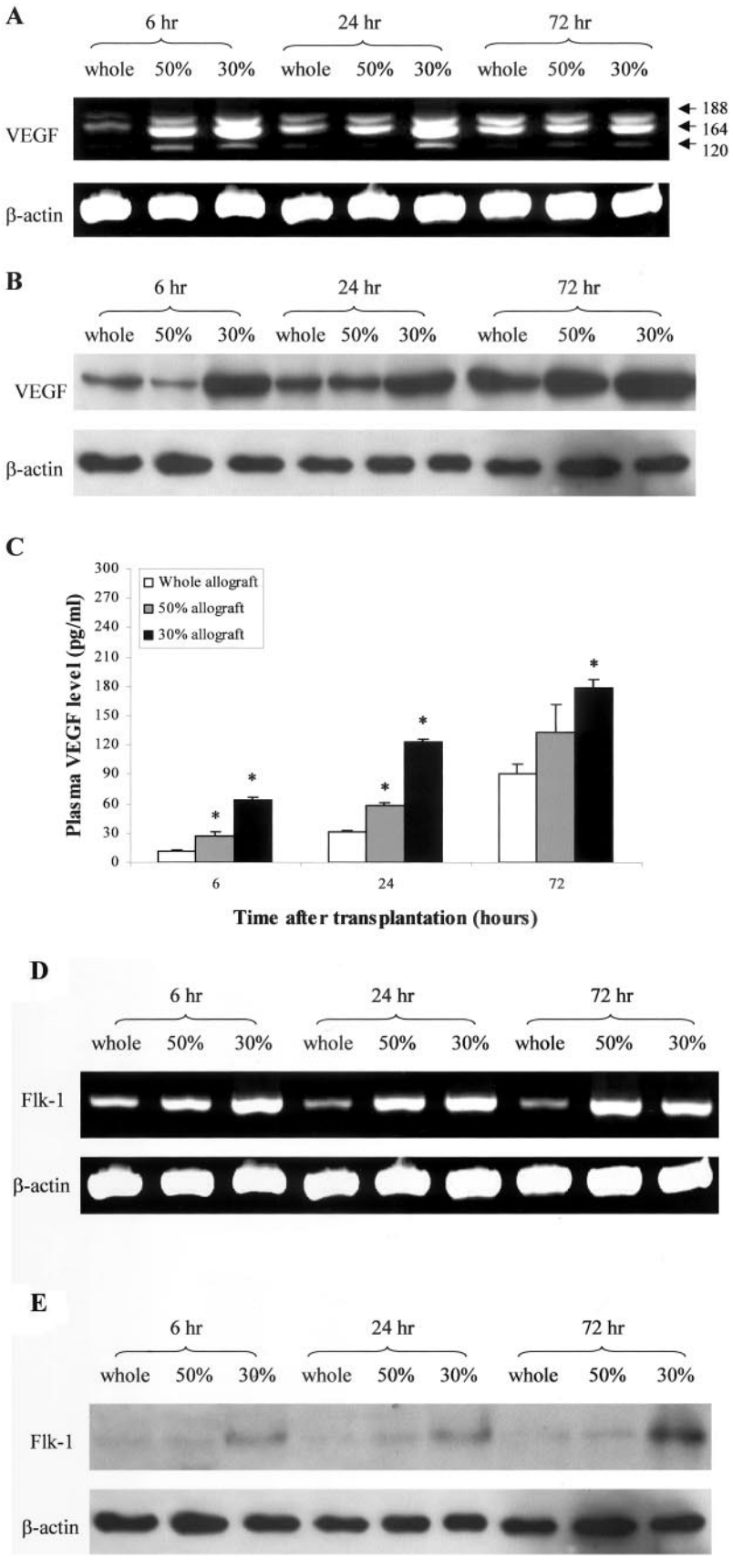

FIGURE 2. VEGF and Flk-1 expression in allografts during the early period after reperfusion. The mRNA (*A*) and protein (*B*) levels of VEGF in 50 and 30% allografts were than the whole allograft. The three bands in *A* represented VEGF isoforms 120, 164, and 188. The soluble VEGF (*C*) levels in plasma were also detected significantly higher in the 50 and 30% allograft groups, especially the 30% allograft group. The mRNA (*D*) and protein (*E*) levels of Flk-1 demonstrated the same phenomenon as VEGF. *, $p < 0.05$, compared with whole allograft group. There were four animals at each time point in each group.

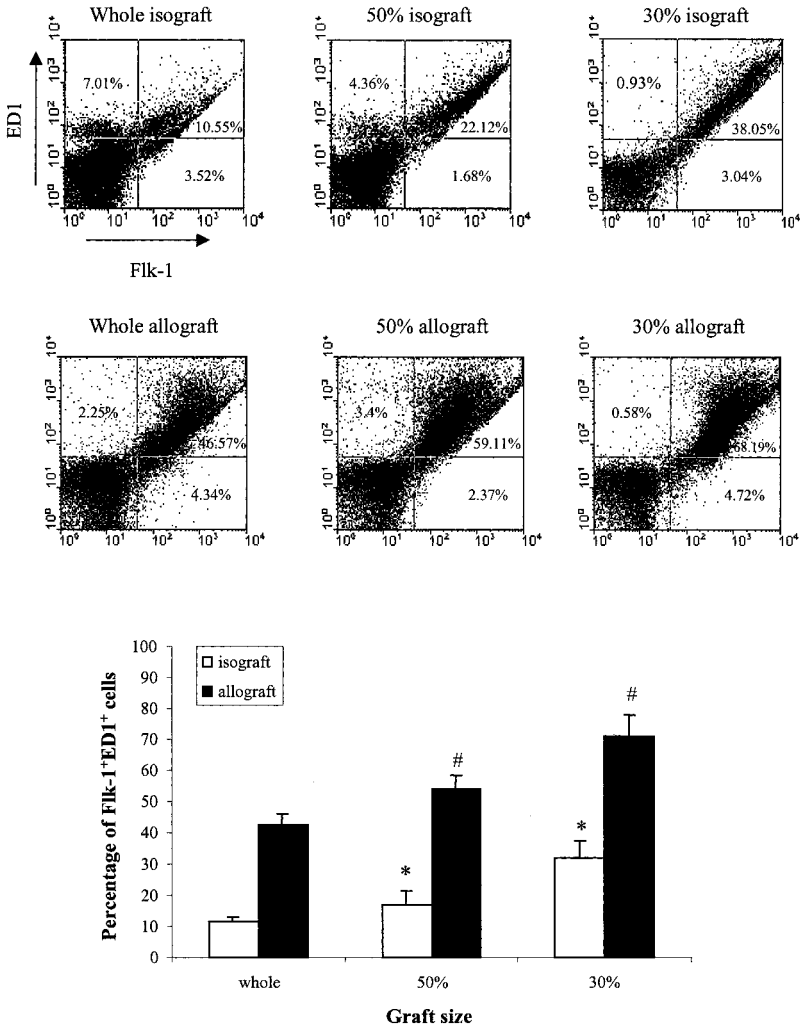

FIGURE 3. Identification of Flk-1 positive (Flk-1^+) macrophages ($ED1^+$) in liver grafts. The mononuclear cells were isolated from liver grafts on day 3 after reperfusion and labeled with Flk-1 and ED1 Abs. A significantly increased number of Flk-1^+ED1^+ cells were detected in both small-for-size isografts (50%, $n = 3$; 30%, $n = 3$) and allografts (50%, $n = 4$; 30%, $n = 4$). *, $p < 0.05$, compared with whole isograft ($n = 3$). #, $p < 0.05$, compared with whole allograft ($n = 4$).

VEGF were designed according to Liu et al. (15). Primer sequences for rat Flk-1 were: sense, 5′-CCAAGCTCAGCACACAAAAA-3′ and antisense, 5′-CCAACCACTCTGGGAACTGT-3′. Protein levels in tissue were determined by standard Western blot analysis using 12% SDS-PAGE gel (monoclonal mouse anti-rat VEGF and polyclonal rabbit anti-rat Flk-1 Abs were purchased from Santa Cruz Biotechnology, Santa Cruz, CA). Soluble VEGF levels in plasma were detected by ELISA according to the protocol suggested by the manufacturer (R&D Systems, Minneapolis, MN).

Cell culture

Cell lines. Macrophage cell lines CRL-2192 were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in F-12K medium with 15% FBS and 1% penicillin-streptomycin (Invitrogen Life Technologies, Carlsbad, CA) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were cultured in serum-free medium for 12 h before different doses of rat VEGF (R&D Systems), 5 μg/ml Flk-1 inhibitor (E)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)amino-carbonyl]acrylonitrile (SU1498; Calbiochem-Novabiochem, San Diego, CA), 10 ng/ml rat IL-1β, 10 ng/ml TNF-α (BioVision, Mountain View, CA), 400 μM desferrioxamine (Sigma-Aldrich, St. Louis, MO), 2 mM sodium salicylate (Santa Cruz Biotechnology), and 1 μg/ml heat shock protein 90 (Hsp90) inhibitor, geldanamycin (Sigma-Aldrich) for 12 h according to treatment regimens.

Primary culture. Mononuclear cells were isolated from the peripheral blood of recipients at 72 h after reperfusion using Ficoll gradient centrifugation. The isolated mononuclear cells (1×10^6) were then treated with 5 ng/ml VEGF, 5 μg/ml SU1498 (Flk-1 inhibitor), and VEGF combined with SU1498, respectively, for 12 h before flow cytometry analysis. Mononuclear cells were also isolated from liver grafts according to the method described by Curry et al. (16) for phenotypic analysis by flow cytometry.

Flow cytometry

Cells from the primary culture were labeled with FITC-conjugated mouse anti-rat ED1 and PE-conjugated mouse anti-rat CD80 or CD86 mAbs (Serotec, Oxford, U.K.), or PE-conjugated mouse anti-rat ED1 (Serotec) and FITC-conjugated rabbit anti-rat Flk-1 Abs (Santa Cruz Biotechnology), and detected in FACSCalibur (BD Immunocytometry Systems, San Jose, CA). Cells from macrophage cell lines were labeled with FITC-conjugated rabbit anti-rat Flk-1 Ab (Santa Cruz Biotechnology) and also detected in FACSCalibur (BD Immunocytometry Systems). Appropriate isotypes of irrelevant Abs were used as controls.

Cell migration assay

Cells from macrophage cell lines were suspended in 50 μl F-12K medium with 1.5% FBS and inoculated on the surface of a polycarbonate filter of a 96-well microchemotaxis chamber (1×10^5 per well; NeuroProbe, Bethesda, MD). In the lower chamber, 299 μl of 15% FBS-F-12K medium, or 1.5% FBS-F-12K medium with either 5 ng/ml VEGF, 5 μg/ml SU1498, or VEGF combined with SU1498 was applied into each well, respectively. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air for 4 h before they were counted under microscope. Five areas were randomly chosen in each well and the number of cells was expressed as mean ± SD.

Statistical analysis

Comparisons of plasma VEGF levels, the number of Flk-1^+ cells in macrophage cell lines or double-positive cells in primary culture between different treatment groups were performed using Student's *t* test (GraphPad, San Diego, CA). Values of $p < 0.05$ were considered as statistically significant.

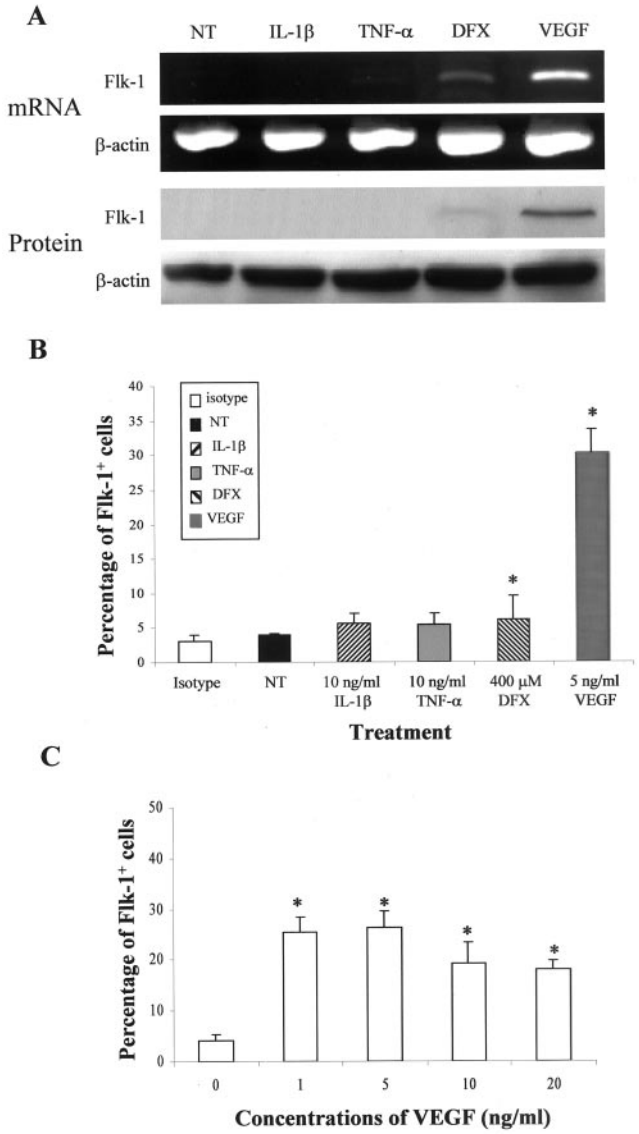

FIGURE 4. Expression of Flk-1 in macrophage cell lines. *A*, The expression of Flk-1 mRNA and protein in macrophages was up-regulated when the cells were treated with desferrioxamine (DFX) and, particularly, 5 ng/ml VEGF, but not proinflammatory cytokines IL-1β and TNF-α. *B*, The number of Flk-1$^+$ cells was also increased after VEGF treatment. *C*, In addition, the number of Flk-1$^+$ cells was related to the doses of VEGF, and the highest number of cells was detected with 5 ng/ml VEGF. NT, no treatment. $*$, $p < 0.05$, compared with NT group. Student's *t* test was performed based on three independent experiments.

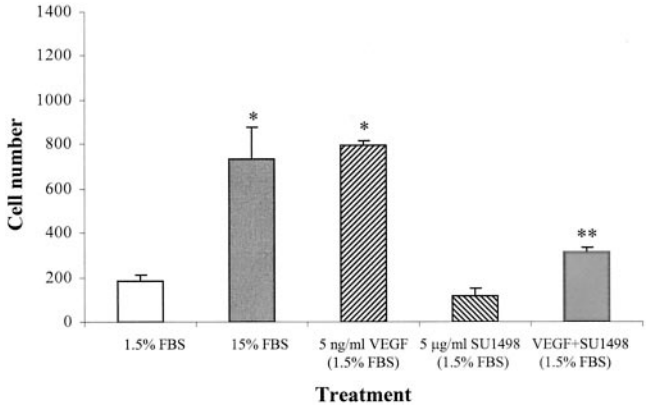

FIGURE 5. The effects of VEGF and Flk-1 on macrophage migration. The macrophage cell lines were cultured with 1.5% FBS, 15% FBS, 5 ng/ml VEGF, 5 μg/ml Flk-1 blocker (SU1498), and VEGF with SU1498, respectively, for 4 h before being counted under microscope. With 15% FBS and 5 ng/ml VEGF, the number of migrated cells was remarkably increased, whereas when SU1498 was administered with VEGF, the migration was significantly suppressed. $*$, $p < 0.05$, compared with 1.5% FBS; $**$, $p < 0.05$, compared with 5 ng/ml VEGF. Student's *t* test was performed based on three independent experiments.

Results

The levels of VEGF and Flk-1 expression in liver grafts were related to graft size

At 6 h after reperfusion, up-regulation of VEGF at both mRNA and protein levels were detected in the small-for-size graft groups in both syngeneic and allogeneic combinations, particularly in 30% grafts (Figs. 1, *A* and *B*, and 2, *A* and *B*). In both 30% isograft and allograft groups, VEGF levels in plasma detected at 6, 24, and 72 h after reperfusion were significantly higher than whole isograft and allograft groups, whereas the increased plasma levels of VEGF in 50% isograft and allograft groups were detected at 6 and 24 h after reperfusion (Figs. 1*C* and 2*C*). In addition, the mRNA and protein levels of Flk-1 were also increased in the small-for-size liver grafts at 6, 24, and 72 h after reperfusion in both isogeneic and allogeneic settings (Figs. 1, *D* and *E*, and 2, *D* and *E*). At 72 h after reperfusion, an increased number of Flk-1$^+$ED1$^+$ cells were detected in the small-for-size isograft and allograft groups, compared with whole isograft and allograft groups (Fig. 3).

Expression of Flk-1 on macrophage was induced by VEGF

By RT-PCR and Western blot, it was found that the macrophage cell lines did not initially express detectable Flk-1 mRNA and protein, but could be induced by desferrioxamine and, preferably, VEGF. However, the expression of Flk-1 on macrophages could not be stimulated by inflammatory cytokines (IL-1β and TNF-α; Fig. 4*A*). The number of Flk-1$^+$ macrophages was increased with the stimulation of desferrioxamine and, especially, VEGF (Fig. 4*B*), and the highest number of Flk-1$^+$ macrophages was induced by 5 ng/ml VEGF. However, with the increased doses of VEGF (10 and 20 ng/ml), the number of Flk-1$^+$ macrophages did not further increase, though it was still significantly higher than the no treatment group (Fig. 4*C*).

Macrophage migration was dependent on VEGF and Flk-1

To identify the role of VEGF and Flk-1 on macrophage migration, a migration assay was performed using VEGF as a chemotaxic attractor. Similar to 15% FBS, 5 ng/ml VEGF in 1.5% FBS-culture medium could induce a significantly higher number of macrophage migration than culture medium with 1.5% FBS only, whereas the migration behavior was inhibited by SU1498 (Flk-1 blocker) administration (Fig. 5).

The activities of monocytes were associated with their expression of Flk-1

To further clarify the role of VEGF and Flk-1 expression on monocyte proliferation (CD80 and CD86 were used as markers to label activated monocytes), mononuclear cells were isolated from the peripheral blood of the recipients at 72 h after reperfusion, and cultured with 5 ng/ml VEGF, 5 μg/ml SU1498, and VEGF combined with SU1498, respectively, for 12 h. In both syngeneic and allogeneic combinations, VEGF could remarkably increase the number of ED1$^+$CD80$^+$ and ED1$^+$CD86$^+$ cells after a 12-h culture in both the whole and small-for-size graft groups. However,

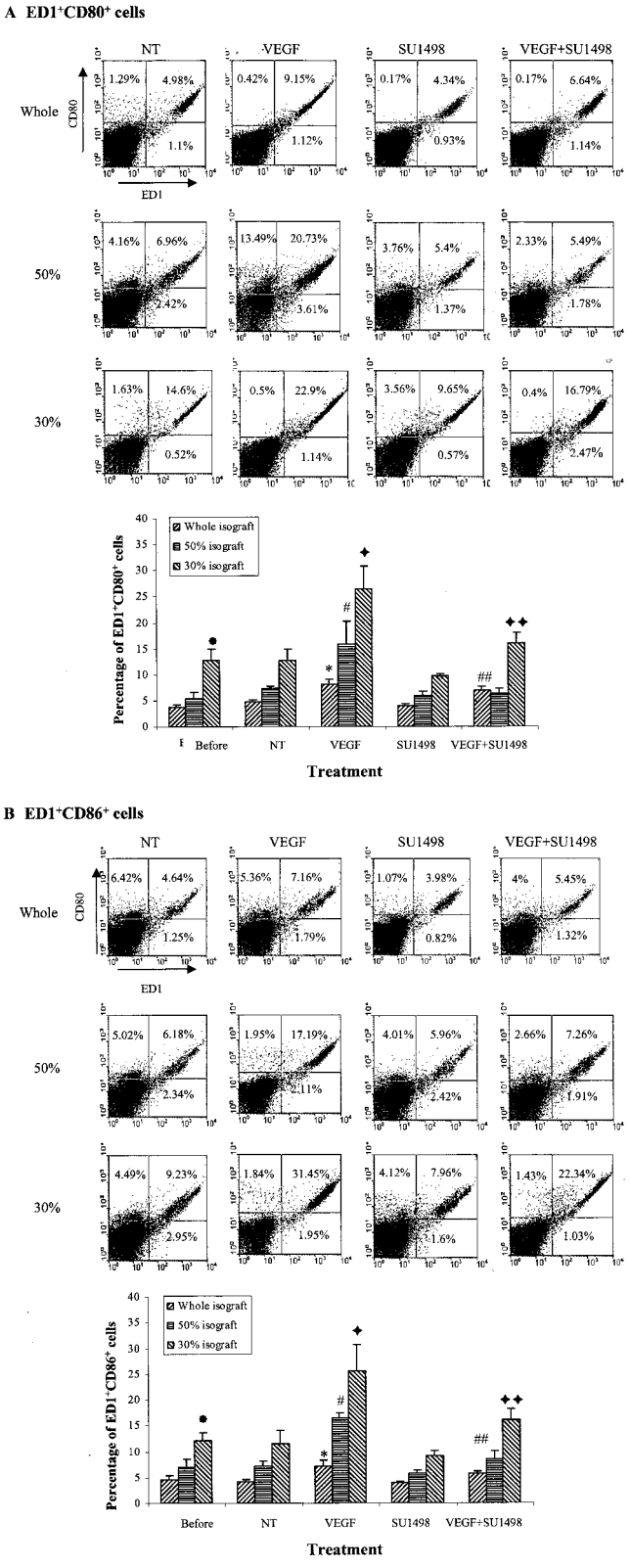

FIGURE 6. The effects of VEGF and Flk-1 on proliferation of activated monocytes in syngeneic transplantation (isolated from recipients' peripheral blood at 72 h after reperfusion). The numbers of ED1$^+$CD80$^+$ (*A*) and ED1$^+$CD86$^+$ (*B*) cells in both whole ($n = 3$) and small-for-size (50% ($n = 3$) and 30% ($n = 3$)) isograft groups were remarkably increased after VEGF stimulation, whereas the increased number of cells could be reduced with SU1498 only in the small-for-size isograft groups, but not in the whole isograft group. NT, no treatment; ⋆, $p < 0.05$, compared with the number of cells isolated from the whole isograft group; ∗, $p < 0.05$, compared with the cell number without treatment (cells from the whole isograft group); #, $p < 0.05$, compared with the cell number without treatment (cells from the 50% isograft group); ❖, $p < 0.05$, compared with the cell number without treatment (cells from the 30% isograft group); ##, $p < 0.05$, compared with the cell number after VEGF culture (cells from the 50% isograft group); ❖❖, $p < 0.05$, compared with the cell number after VEGF administration (cells from the 30% isograft group).

the increase of ED1$^+$CD80$^+$ and ED1$^+$CD86$^+$ cells was reduced when SU1498 was administered with VEGF in all the small-for-size graft groups and the whole allograft group, but not the whole isograft group (Figs. 6 and 7).

The inducible expression of Flk-1 on macrophages was related to NF-κB and Hsp90

To explore the possible molecules that might be related to Flk-1 expression on macrophages, the nonsteroid anti-inflammatory drug, sodium salicylate, and the Hsp90 inhibitor, geldanamycin, were introduced to macrophage cell lines with VEGF. It was found that VEGF could augment the NF-κB (*p*65) protein level, but it had no obvious effect on Hsp90. However, administration of sodium salicylate with VEGF could reduce the levels of both *p*65 and Hsp90. Similarly, administration of geldanamycin to VEGF could also decrease the level of Hsp90 and reverse the up-regulated *p*65 level induced by VEGF (Fig. 8, *A* and *B*). At the same time, the increased number of Flk-1$^+$ cells induced by VEGF was completely reversed by sodium salicylate, and partially by geldanamycin (Fig. 8*C*).

Discussion

Our previous studies demonstrated that the inflammatory status and acute rejection process were exacerbated in the small-for-size allografts, compared with whole allografts and small-for-size isografts (5, 17), and there might be a linkage between injury, inflammation, and acute allograft rejection in these small-for-size allografts. In the present study, we detected a significantly increased production of VEGF in small-for-size liver transplantation during the early phase after reperfusion, and hepatocytes were the major source of VEGF in these liver grafts (data not shown). The augmentation of VEGF in both isogeneic and allogeneic settings indicated that the microcirculatory injury due to hemodynamic changes in the small-for-size liver grafts, as demonstrated previously by our group (1, 18), might contribute to the induction of VEGF up-regulation. This was similar to the finding of Boros et al. (19) that VEGF expression might augment after long-term preservation, in which ischemia/reperfusion injury occurred. The increased expression of VEGF stimulated endothelial cell proliferation and migration, which helped liver graft regeneration (6), the process that happened several hours after reperfusion (20). In contrast, an even higher level of VEGF expression in the small-for-size allografts than the small-for-size isografts implied the potential role of this molecule in mediating inflammation and acute allograft rejection, as the current study also revealed that VEGF might function as a chemotaxic mediator that could enhance macrophage activities.

The interaction between VEGF and its receptors plays a crucial role in mediating monocyte and macrophage activities. It was reported by Sawano et al. (10) that human monocytes only expressed VEGF-R1 (fms-like tyrosine kinase-1), but not Flk-1. However, in the situation of small-for-size liver transplantation, we did find a group of cells that expressed both macrophage marker-ED1 and Flk-1 in both isografts and allografts, suggesting that macrophages might express Flk-1 in certain circumstances. However, we did not

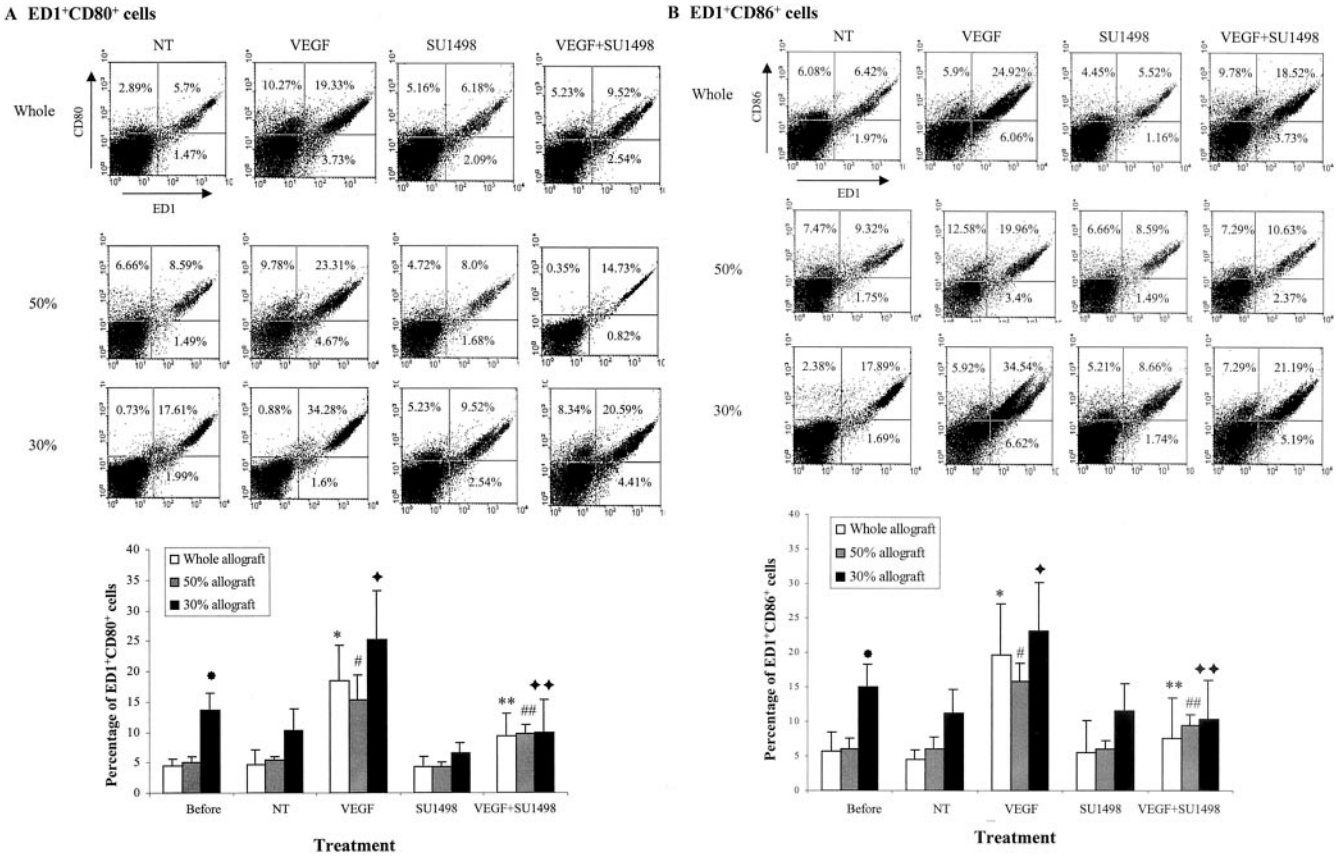

FIGURE 7. The effects of VEGF and Flk-1 on proliferation of activated monocytes in the allogeneic setting (isolated from recipients' peripheral blood at 72 h after reperfusion). The numbers of ED1$^+$CD80$^+$ (*A*) and ED1$^+$CD86$^+$ (*B*) cells in both whole ($n = 4$) and small-for-size (50% ($n = 4$) and 30% ($n = 4$)) allograft groups were remarkably augmented after VEGF stimulation, whereas the increased number of cells could be reduced with Flk-1 blockade. NT, no treatment; ⋆, $p < 0.05$, compared with the number of cells isolated from the whole allograft group; ∗, $p < 0.05$, compared with the cell number without treatment (cells from the whole allograft group); #, $p < 0.05$, compared with the cell number without treatment (cells from the 50% allograft group); ✦, $p < 0.05$, compared with the cell number without treatment (cells from the 30% allograft group); ∗∗, compared with the cell number after VEGF treatment (cells from the whole allograft group); ##, $p < 0.05$, compared with the cell number after VEGF culture (cells from the 50% allograft group); ✦✦, $p < 0.05$, compared with the cell number after VEGF administration (cells from the 30% allograft group).

detect Flk-1 expression in macrophage cell lines when no treatment was given. When the cells were stimulated by iron chelator desferrioxamine (desferrioxamine could induce endogenous VEGF production in various types of cells through generating a hypoxic condition; Refs. 21 and 22) and exogenous VEGF, an increased expression of Flk-1 was detected preferably by the exogenous VEGF. Additionally, the inducible expression of Flk-1 on macrophages by VEGF displayed a dose-dependent manner and could reach a peak level with the dose of 5 ng/ml, implying that Flk-1 might be an inducible receptor on macrophages, and exogenous VEGF stimulation might function more effectively on Flk-1 expression. However, we did not observe any noticeable Flk-1 changes in the liver grafts at different time points after reperfusion, during which an increased expression of VEGF was detected. This might be because macrophages were not the only cell population that expressed Flk-1, and more abundant Flk-1 expression was found to be expressed in endothelial cells (23) and hepatic stellate cells (21). Whether the expression of Flk-1 in these types of cells can be augmented by VEGF remains to be determined.

The processes of cell migration and proliferation were two key steps of macrophage activation. It was reported that VEGF could induce monocyte migration in an fms-like tyrosine kinase-1-dependent manner (10). The present study further demonstrated that macrophage migration mediated by VEGF might also depend on Flk-1 expression. This was supported by the evidence that the number of migrated cells by VEGF could be partially reversed with Flk-1 inhibition. However, the question of which of the two VEGF receptors plays a more predominant role needs to be determined. In addition, the expression of Flk-1 on monocytes also contributed to the proliferation of activated monocytes isolated from the peripheral blood of liver graft recipients, as the increased number of CD80$^+$ and CD86$^+$ monocytes by VEGF could be partially reversed by Flk-1 blockade, indicating that VEGF-stimulated monocyte proliferation was partially dependent on Flk-1 expression. The only exception was in the whole syngeneic transplantation. Although we could also observe an increased number of CD80$^+$ and CD86$^+$ monocytes after VEGF stimulation, the augmentation was not inhibited by Flk-1 blockade, indicating that the number of activated monocytes at the time of VEGF stimulation might also be important for the effects of Flk-1. In other words, the effects of VEGF-Flk-1 interaction on monocyte proliferation required a certain number of activated cells. However, further studies are needed to clarify the potential mechanisms. Because several other factors (e.g., proinflammatory cytokines) might also contribute to the activation of monocytes and macrophages, and a significantly increased expression level of VEGF in allografts was detected only after 6 h, it further suggested that the most important role of VEGF on macrophages and monocytes was enhancement of their migration and proliferation activities rather than initiation.

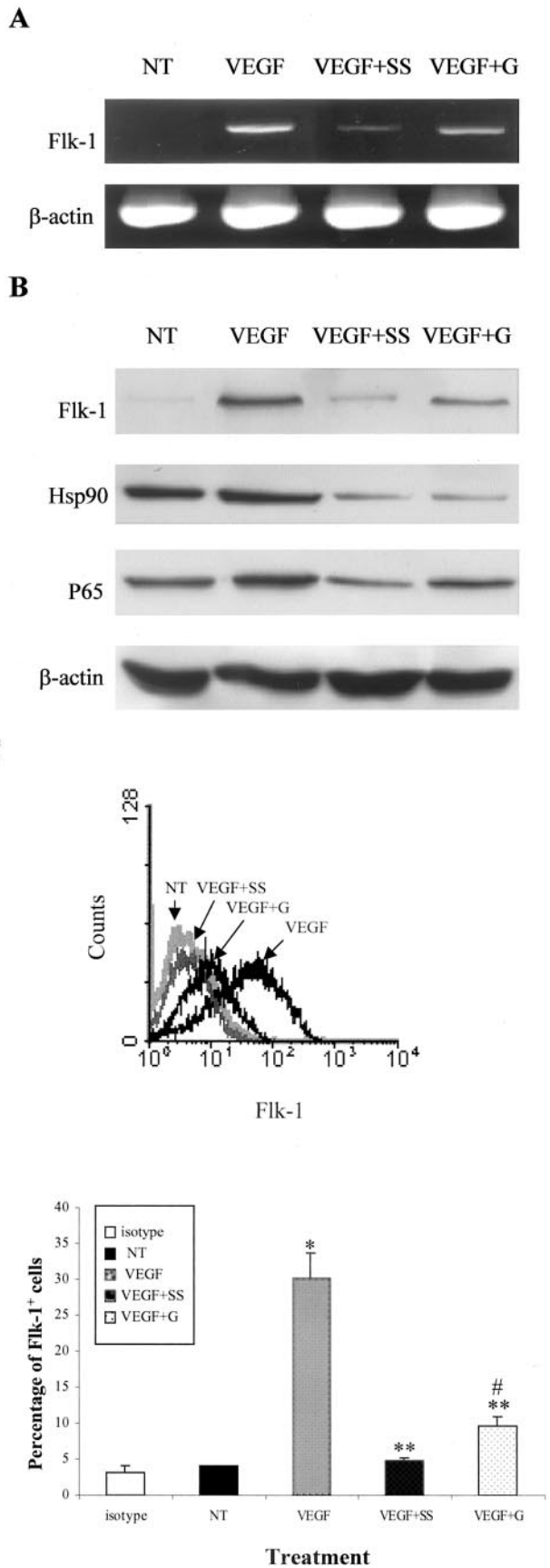

FIGURE 8. Determination of the potential roles of NF-κB (*p*65) and Hsp90 in Flk-1 expression in macrophage cell lines. The up-regulation of Flk-1 at both mRNA (*A*) and protein (*B*) levels by VEGF could be signif icantly decreased by sodium salicylate and geldanamycin. At the same time, reduced expression of *p*65 and Hsp90 was also observed when VEGF was administered with sodium salicylate and geldanamycin. *C*, The increased number of Flk-1^+ cells could be completely reversed by sodium salicylate, and partially by geldanamycin. NT, no treatment; SS, sodium salicylate; G, geldanamycin. *, $p < 0.05$, compared with NT; **, $p < 0.05$, compared with VEGF. Student's *t* test was performed based on three independent experiments.

Finally, we demonstrated in the present study that up-regulation of Flk-1 on macrophages by VEGF was associated with the expression of NF-κB (*p*65) and Hsp90. It has been reported that VEGF could activate NF-κB in a cell type-dependent manner (24), and the activity of NF-κB was related to the activation of the Flk-1 promoter (25). Therefore, introduction of VEGF to macrophages might induce the activation of Flk-1. Our study revealed that the up-regulation of Flk-1 on macrophages by VEGF could be reversed by administration of sodium salicylate. At the same time, a down-regulation of *p*65 was detected in these cells, further suggesting that the activation of Flk-1 was associated with the activity of NF-κB. Hsp90 was found to be crucial in determining leukemia cell survival induced by VEGF (26). In addition, Hsp90 was an important chaperone of several molecules including the tyrosine kinase family, which played crucial roles in the signal transduction cascade that mediated inflammatory activities of mast cells (27). Hsp90 was also found to be able to enhance the activities of inducible nitric-oxide synthase (28) and cytokine production of macrophages (29). Our study suggested that the induction of Flk-1 activation by VEGF was also related to Hsp90, as the increased number of Flk-1^+ macrophages could be partially reduced by the Hsp90 inhibitor, geldanamycin. Further studies are needed to explore the potential mechanism.

In conclusion, our study revealed that VEGF production and VEGF receptor expression were increased in the small-for-size liver grafts during the early period after reperfusion, and up-regulated VEGF expression might enhance monocyte and macrophage activities in a VEGF-R2 (Flk-1)-dependent manner.

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