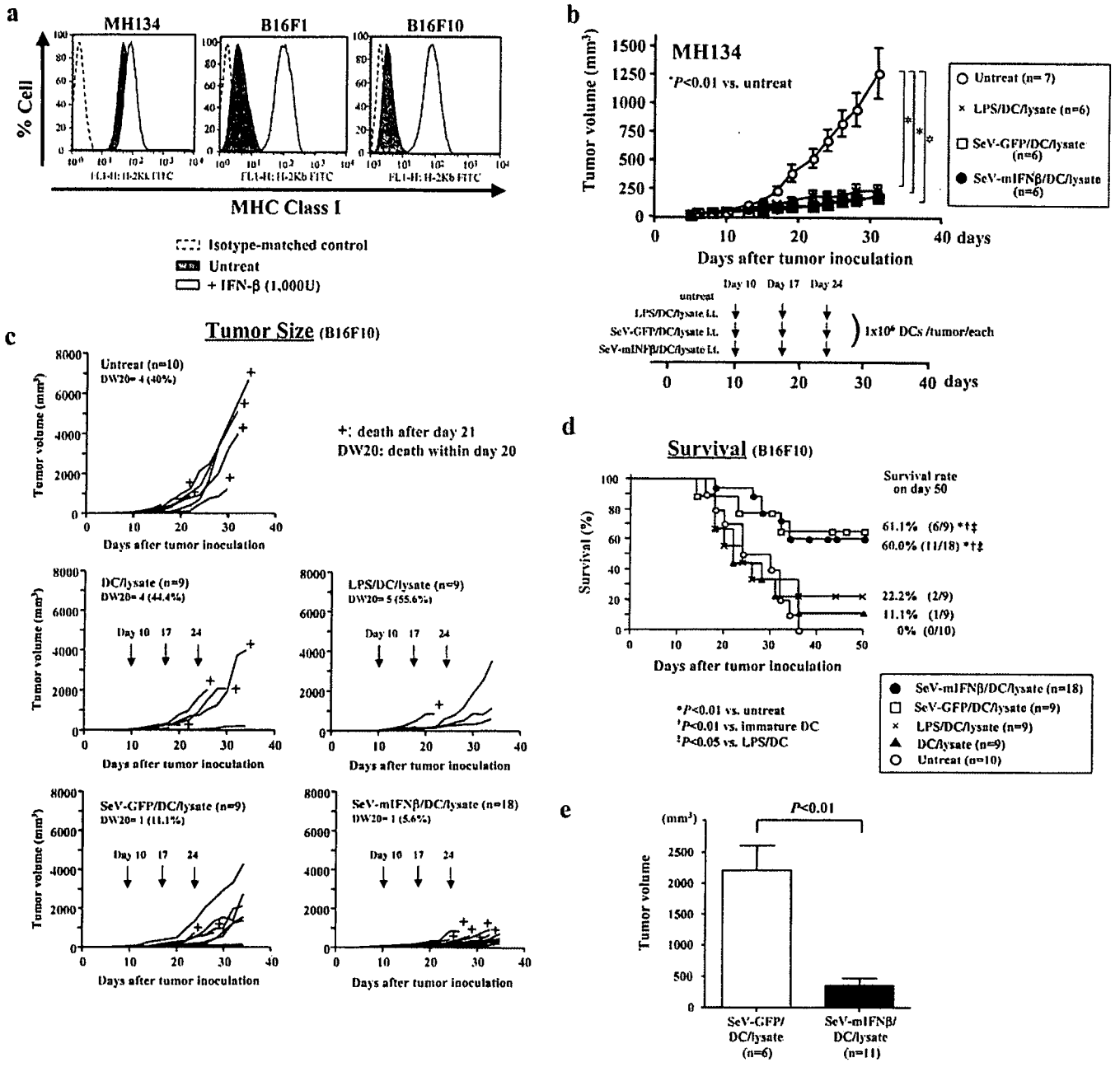


**FIGURE 4.** Tumor cell-dependent, divergent effects of SeV/DC immunotherapy expressing mIFN-β (late treatment regimen). *, $p < 0.01$. *a*, Flow cytometry analysis for MHC class I expression level of tumors treated with or without mIFN-β. After 48-h cultivation of tumor cells (MH134; H-2$^k$ H-2$^k$, and B16F1, B16F10 melanoma; H-2$^b$) with or without mIFN-β, cells were subjected to the analysis. Note that the expression of MHC class I in both melanoma cells was very low, findings that were strongly up-regulated by mIFN-β treatment. *b*, Antitumor effect of SeV/DCs on a MH134 tumor, which was resistant to mIFN-β, with or without expression of mIFN-β in vivo. C3H/HeN mice were inoculated intradermally with $1 \times 10^6$ MH134 cells, which were resistant to mIFN-β, on day 0, and DC treatment was started at day 10 using $10^6$ cells of LPS/DC (×), SeV-IFN-β/DC (●) or SeV-GFP/DC (□), as indicated in the bottom scheme. The data contains the results of all animals in two independent experiments. *c*, Time course of the volume of individual B16F10 tumors, a IFN-β-sensitive malignancy, treated with DC/lysate, LPS/DC/lysate, SeV-GFP/DC/lysate, or SeV-mIFN-β/DC/lysate. Untreated animals were used as a control group. DW20 indicates the number of death animals within day 20. +, Individual animals with death after day 21. *d*, Survival curve of the mice bearing B16F1 melanoma treated with various DCs. Significant prolongation of survival of mice bearing B16F10 melanoma was seen in SeV/DC groups, but not others. Data demonstrated were the total of four independent experiments. *e*, A graph indicating the effect of exogenous mIFN-β on tumor size of B16F10 melanoma. SeV/DC-treated animals surviving over 36 days were evaluated.

*Immunological assessments*

To explore the immunological effectors for the antitumor effects in DC therapy as well as gene therapy, we next focused on NK cells and CTL activities in mice bearing B16F10 and MH134 tumors treated by each method.

NK cells obtained from mice bearing B16F10 tumors treated with DCs, including LPS/DCs, SeV-GFP/DCs, and SeV-mIFN-β/DCs, did not show significant cell lysis activity. In contrast, NK cells from mice treated with direct i.t. vector injection of SeV-mIFN-β, aforementioned in Fig. 3*b*, showed apparently

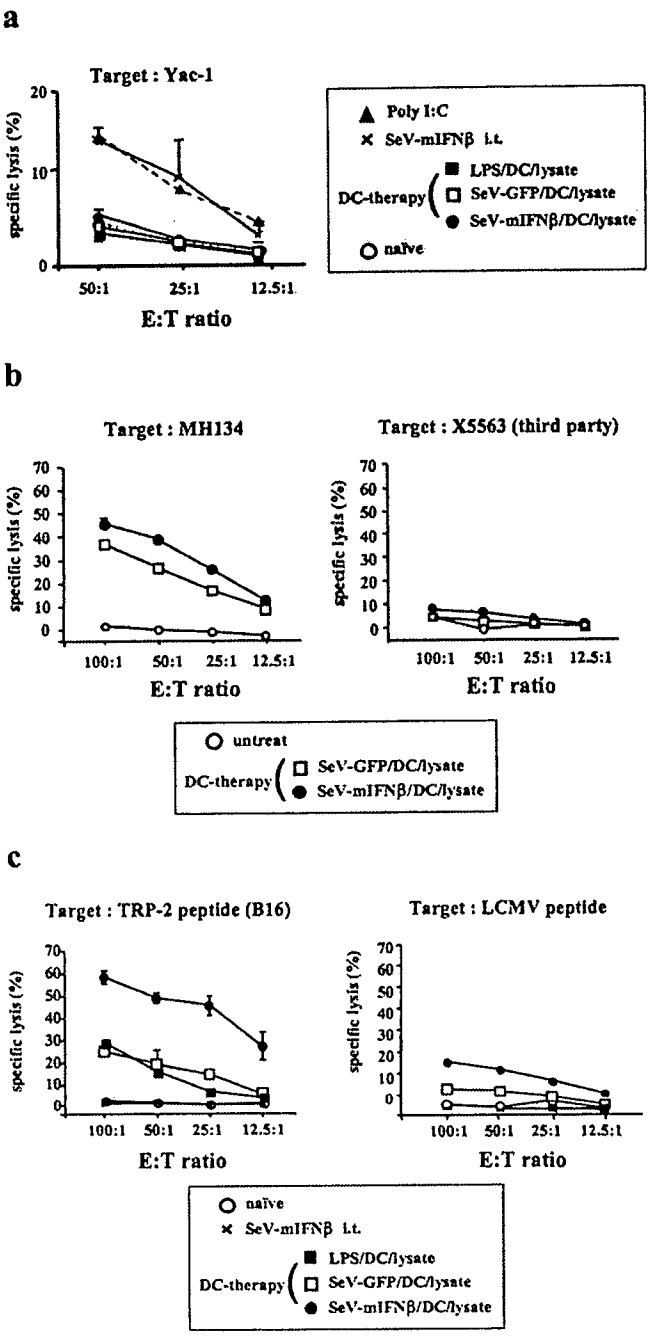


**FIGURE 5.** Effectors contributing to the antitumor effect of SeV/DC immunotherapy. *a*, Assessment of NK cell activity in B16F10 melanoma. Thirty-one days after B16F10 cell inoculation, splenocytes were isolated from the mice treated with SeV-GFP/DC/lysate (□), SeV-mIFN-β/DC/lysate (●), LPS/DC/lysate (■), or intratumoral direct injection of SeV-mIFN-β vector (X). The cytolytic function against $^{51}$Cr-labeled YAC-1 targets was assessed by $^{51}$Cr release. Splenocytes from the mouse, which were treated with 150 μl of poly I:C 24 h before the assay, were used as a positive control. *b*, Assessment for CTL activity of MH134. Induction of tumor-specific CTLs after i.t. administration of SeV-GFP/DC/lysate (□), SeV-mIFNβ/DC/lysate (●), and LPS/DC/lysate (■), which was repeated three times every week according to the late treatment regimen. Controls included tumor-bearing mice without any treatment (○). X5563 was also used as a target of a third party. Seven days after the last treatment, splenocytes were isolated and restimulated in vitro for 5 days with mitomycin C-treated MH134 cells, and cytolytic activity against $^{51}$Cr-labeled targets was measured. The figure shows results from one of three similar experiments. *c*, Assessment for CTL activity of B16F10 cells. Induction of tumor-specific CTLs after i.t. administration of SeV-GFP/DC/lysate (□), SeV-mIFN-β/DC/lysate (●), and LPS/DC/lysate (■), which was repeated three times every week according to the late treatment regimen. Controls included tumor-bearing mice without any treatment (○). LCMV peptide was also used as a target of a third party. The method was same as above. *d*, Determination of immune cell subsets against MH134 or B16F10 responsible for the protective immunity induced by SeV-mIFN-β/DCs was Ab-mediated via an in vivo depletion analysis, as described in *Materials and Methods*. These bar graphs show the tumor volume on day 30 after inoculation of tumor cells. Anti-CD4 (GK1.5), anti-CD8 (53-6.72), or anti-asialo GM1 was i.p. injected according to the indicated schedule. In all animals, >98% of specific depletion of target cells in the spleen and lymph nodes was confirmed by flow cytometry (data not shown). The data contains all animals of two or three separate experiments.

strong cell lysis activity which was comparable to a positive control level (poly I:C) (Fig. 5*a*). These findings indicate that NK cells do not significantly contribute to the antitumor effect of DC therapy.

In contrast, CTLs from the splenocytes of mice showed opposite results, and demonstrated the comparable findings suggested in Fig. 4. In the case of CTLs against MH134 tumor cells, the expression of mIFN-β did not significantly affect the CTL activities induced by SeV/DC therapies (Fig. 5*b*, *left graph*). As a control experiment, X5563 tumor cells were used as a target, and showed negative result (Fig. 5*b*, *right panel*). In the case of TRP-2 peptide, a tumor-specific Ag of B16 melanoma as a target of CTLs obtained from spleens from mice bearing B16F10 melanoma, SeV/DCs showed a similar level of CTL activity compared with that seen in LPS/DCs, a finding that was markedly enhanced by SeV/DCs expressing mIFN-β (Fig. 5*c*, *left panel*). In this case, no significant CTL activity was detected in mice treated with direct vector injection of SeV-mIFN-β. In contrast, a relatively low level of background release was observed when third-party target (LCMV) was used (Fig. 5*d*, *right panel*).

These results thus indicate the significant contribution of IFN-β to the CTL activity for an IFN-β-sensitive B16F10 tumor but not for an insensitive MH134 tumor during DC therapy. Furthermore, these data suggest the distinct mechanisms of the antitumor effect of DC therapy compared with that of gene therapy, even when the same therapeutic gene, IFN-β, was used.

### *Effector cell subsets in DC therapies*

To make it clear which subset(s) of cells is important for the antitumor effect of SeV-mIFN-β/DC therapy, we next conducted the effector cell depletion experiment by administration of each depletion Ab against CD8, CD4, and NK (by asialo-GM1) in mice with MH134 or B16F10 tumors treated with or without SeV-mIFN-β/DCs. The dose of each Ab was determined by our repeated preliminary experiments of FACS analyses that showed >98% of the target subject in some lymph nodes and the spleen (data not shown). The same lot of each Ab

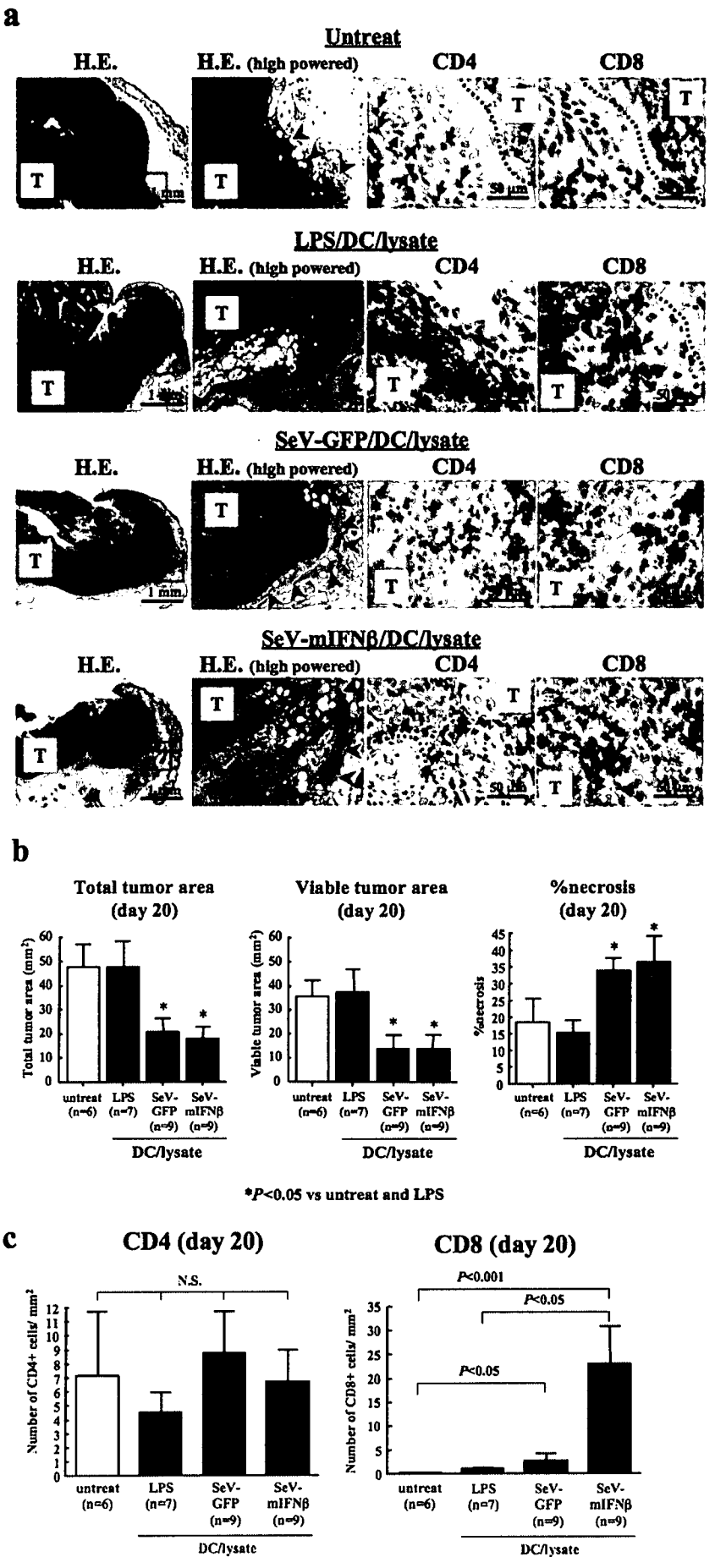


**FIGURE 6.** Histopathological and immunohistochemical examination of B16F10 tumors on day 20. Dot lines indicate the margin of tumors. *a*, Typical representative findings of the tumor environment on 20 days after inoculation of B16F10 treated with or without LPS/DC/lysate, SeV-GFP/DC/lysate, and SeV-mIFN-β/DC/lysate on days 10 and 17. Panels of H&E staining indicated as "high powered" are high powered view of their corresponding *left panels*. Arrowheads indicate the infiltration of chronic inflammatory cells, including lymphocytes and macrophages. Immunohistochemical positive reaction (red cytoplasm) of $CD4^+$ and $CD8^+$ cells are also demonstrated (arrows). These are representative of two separate studies using three to five mice per group. *b*, Bar graphs indicating the squares of total tumor area (*left*), viable tumor area (*middle*), and the ratio of necrotic area (*right*). H&E-stained sections were subjected to the computer-assisted square measurement. *c*, Bar graphs indicating the number of immunohistochemically $CD4^+$ and $CD8^+$ cells per $mm^2$ in the viable tumor area and peripheral tumor tissue.

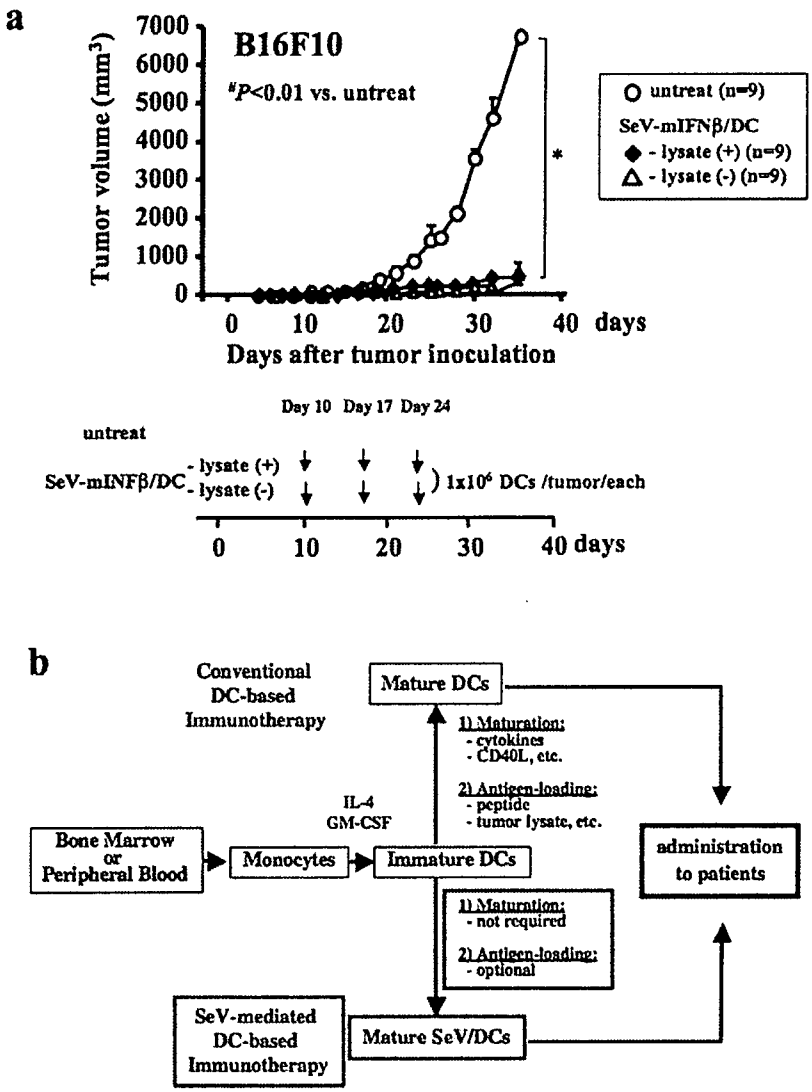


**FIGURE 7.** *a*, Requirement of ex vivo pulsation of tumor lysate during SeV-modified DC immunotherapy. The late treatment regimen against B16F10 melanoma was done via intratumor injection of SeV-mIFN-β/DC with or without tumor lysate. Ten days after B16F10 cell inoculation, i.t. injection of SeV-mIFN-β/DC pulsed with (◆) or without tumor lysate (△) was performed according to the protocol as indicated below. *, $p < 0.01$. *b*, Schematic representation of the comparison of conventional DC-based immunotherapy and that using SeV-modified DCs. The greatest advantages of the SeV/DC method are that: 1) there is no requirement of a specific stimulus to induce strong DC activation representatively, and 2) Ag-loading is not critical when SeV/DC is administered directly to tumors.

was used throughout the experiments. The tumor size was evaluated every day, and the data on day 30 are presented.

As shown in Fig. 5*c*, in the case of MH134 tumors, as expected, depletion either of $CD4^+$ T cells or $CD8^+$ T cells almost totally abrogated the antitumor effect induced by SeV-mIFN-β/DCs, and the depletion of NK cells partly canceled the effect (Fig. 5*c*, *left graph*). These findings indicate that the tumor-specific CTL as well as the helper function of CD4 are absolutely necessary to the therapeutic effect of SeV-mIFN-β/DCs, and further, NK cell activity is also involved in the effect.

In the case of B16F10 melanoma, in contrast, such depletion studies demonstrated unexpected results. Depletion of $CD8^+$ T cells during therapy partially cancelled the protective immunity induced by SeV-mIFN-β/DCs; however, mice depleted of NK cells during therapy showed no significant effect (Fig. 5*c*, *right graph*), data supported by the cell lysis analysis shown in Fig. 5*a*. When $CD4^+$ T cells were depleted, however, the antitumor effect of SeV-mIFN-β/DCs was markedly and significantly enhanced, indicating that the net effect of $CD4^+$ T cells was to function as a helper for tumors rather than providing antitumor immunity. These data might possibly reflect the function of regulatory T cells ($CD4^+$ $CD25^+$ T cells) in the $CD4^+$ T cell subset (30).

These findings confirm that $CD8^+$ T cells were the ubiquitous and predominant effector cells in antitumor immunity for both MH134 and B16 melanoma, even though their quantitative contribution may vary depending on the tumor type.

*Histological and immunohistochemical assessments for modification of tumor environment*

Next, we histopathologically examined the B16F10 melanoma tumor environment, including tumor area (viable tumor and ratio of necrosis) and infiltration of CD4 and CD8 T lymphocytes, using another set of DC therapy experiments. At that time, DC therapy was done on days 10 and 17, and the tumor was harvested on day 20. Four of 10 animals of the untreated group and 3 of 10 of the LPS/DC group but not all SeV/DC groups were dead within day 20.

Histological examination suggested that the infiltration of inflammatory cells around tumor was more pronounced in DC therapy groups (Fig. 6*a*), and interestingly, more reduction of tumor area and viable tumor area was seen in the SeV/DC groups, but not in the LPS group (Fig. 6*b*, *left* and *middle graphs*). In contrast, the ratio of tumor necrosis was more pronounced in the SeV/DC groups (Fig. 6*b*, *right graph*), and these findings were observed irrespective of use of SeV/DCs expressing IFN-β.

Immunohistochemistry demonstrated more frequent infiltration of CD8 T lymphocytes into tumor and surrounding s.c. tissue by treatment of SeV-mIFN-β/DCs compared with other

groups (Fig. 6*c*, *right graph*), a comparable finding obtained in Fig. 5*c*, while no significant difference was determined in CD4 T lymphocyte infiltration among all groups tested (Fig. 6*c*, *left panel*).

These results thus suggest the split mechanisms of SeV-mediated modulation of DC function and expression of IFN-β on the antitumor effect of B16F10 melanoma.

*No requirement of Ag loading during ex vivo culture of SeV-mIFN-β/DCs*

Finally, we investigated the requirement of ex vivo Ag loading during the culturing of SeV-mIFN-β/DCs, because ex vivo Ag loading has been considered to be an essential step in inducing tumor-specific immunity (31, 32). To assess this, we used a late treatment regimen against B16F10 melanoma via i.t. injection of SeV-mIFN-β/DC with or without tumor lysate.

As shown in Fig. 7, the therapeutic effect of SeV-mIFN-β/DCs was not affected by ex vivo Ag-loading when DCs were injected i.t., indicating that the antitumor effect can be led by direct i.t. injection of SeV-mIFN-β/DCs without any supplementation of ex vivo Ags.

## Discussion

During the last decade, clinical trials of DC-based immunotherapy have revealed a relatively limited clinical outcome against intractable malignancies (3). These early results do not always suggest the limited potential of DC-based immunotherapy, however, because there are still a number of issues to be clarified, including how to monitor the activation of DCs, which route and with what frequency DCs should be administered, how Ags should be targeted, and which malignancy and what stages of malignancy should be selected (2). Physicians and scientists should clarify these points and apply the answers to the basic mechanisms of tumor biology and DC therapy, because no one knows, at present, the optimized potentials of DC-based cancer immunotherapy in clinical settings.

We here assessed the antitumor potential of a novel DC-activating modality, rSeV, and directly compared it to that of LPS, which is not relevant clinically but experimentally shows the high performance in terms of DC activation. Key observations obtained in this study were as follows: 1) SeV not only transduced foreign genes to immature DCs but also led them a highly activated state that was comparable to that induced by LPS, 2) the bioactive potential of SeV/DCs as an antitumor agent was seen in tumor-bearing mice in vivo, 3) complete elimination of an established low malignancy tumor, IFN-β-sensitive B16F1, could be found via an early treatment regimen using SeV-GFP/DCs, findings enhanced by SeV/DCs expressing mIFN-β, 4) SeV/DC therapy was likely to be more effective than protein and gene therapies, 5) distinct mechanisms of antitumor effects were suggested between gene therapy and SeV/DC therapy, even though both used the same therapeutic gene, mIFN-β, and 6) the process of ex vivo Ag loading was not important in the therapeutic effect of SeV-mIFN-β/DCs when they were injected i.t.

Because this is the first demonstration of a negative strand RNA virus-mediated DC activation for cancer immunotherapy, there are still a number of unanswered questions regarding this system. However, it should be true that SeV is a strong and important modality for activating DCs which may be useful for cancer immunotherapy.

At present, the precise molecular and cellular mechanisms of activation of DCs by SeV are not well-understood. In this study, we demonstrated that DCs treated by SeV secrete several inflammatory cytokines, including IL-1β, IL-6, and TNF-α, that are strong activators to DCs in mixture (33); however, no knowledge of how DCs exposed to SeV express these cytokines is available at present. A recent important report indicated that DCs treated with SeV produce multiple proinflammatory cytokines independent of TLRs and their adaptors, including MyD88 (34), unlike LPS, CpG (35), or OK-432 (36), which stimulate DCs through a TLR-dependent mechanism.

An important finding obtained in the current study is that stronger antitumor effect on B16 melanomas was seen in SeV-modified DCs compared with that seen in LPS/DCs, even though the expression of costimulatory molecules and typical Th1 cytokines of SeV/DCs did not always reach the levels of LPS/DCs. These results strongly suggest the complex mechanism of antitumor effect; namely, antitumor activity of DC immunotherapy cannot generally be predicted by the expression of these molecules. We are now assessing this point extensively.

An essential finding in the current study is that SeV-mIFN-β/DC therapy was considerably effective for treating IFN-β-sensitive tumors, B16 melanomas, even in the case of the highly malignant B16F10 subtype. More importantly, this effect was shown by the later treatment regimen, on day 10, when the tumor was 7–9 mm in diameter and well-vascularized as revealed by histopathological assessment (data not shown). Because "there are no cancer vaccine models that reproducibly demonstrate that vascularized tumors can be rejected" at present, as noted by Rosenburg et al. (3), SeV-mIFN-β/DC therapy may be an important candidate for overcoming the current limitation in immunotherapy for melanoma in the clinical setting.

An important question raised in this study is what is the precise mechanism(s) of SeV-mIFN-β/DC therapy against B16F10 melanoma? Fig. 5 suggested that NK cells seemed not to be important in this case, while they did actually show the antitumor effect seen in MH134, at least in part. A $CD8^+$ cell depletion study demonstrated $CD8^+$ CTLs as major effectors for SeV-mIFN-β/DC therapy for B16F10 melanoma, and mIFN-β expression in DCs enhanced the CTL activity (Fig. 5*b*). These findings show that the direct cytotoxicity of mIFN-β and the induction of $CD8^+$ CTLs may be major players in the antitumor effect of SeV-mIFN-β/DC therapy against B16F10 melanoma, which may be modified by the expression of MHC class I in tumor cells. This explains why the antitumor effect against MH134, which was insensitive to mIFN-β, was not modified by the use of SeV-mIFN-β/DCs. Modulation of MHC class I expression is an important part of antitumor immunity; loss or down-regulation of MHC class I is an important mechanism for the tumor's escape from the host immune system, resulting in the peripheral tolerance (37–39).

In addition, it is of interest that our current study demonstrated that distinct beneficial effect of SeV/DCs and those expressing exogenous IFN-β; namely, modification of DC function by SeV contributed to the survival and increase of the tumor necrotic area, and exogenous expression of mIFN-β significantly enhanced CTL activity and increased recruitment of $CD8^+$ T cells. These results thus suggest the modulation of host immune response to malignancies via DC immunotherapy by mIFN-β; however, exact mechanisms of apparent improvement of survival and increase of necrotic area via SeV/DCs without exogenous gene, that was not seen by LPS/DCs, are still unknown. One possible explanation is the effect caused by DC-derived endogenous type I IFNs, which were not detected by LPS treatment, caused by viral transduction. According to our careful observation, death of mice bearing B16F10 within day 20 was caused by extensive hemorrhage from ulceration of tumors. It has been shown that low-dose type I IFNs,

including IFN-$\beta$, reduced tumor vasculature, a possible mechanism of reduced rate of hemorrhage. This is likely because some previous studies demonstrated that the antiangiogenic activity of mIFN-$\beta$ did not show apparent dose response (40), a comparable finding obtained in the current study. Such multiple functions of IFN-$\beta$ would be favorable for cancer immunotherapy, further studies should be done to assess the precise mechanism of action of IFN-$\beta$ in clinical settings.

One more question raised in this study involves the role of $CD4^+$ T cells in antitumor immunity, which showed conflicting results between two different tumors, namely, depletion of $CD4^+$ cells completely canceled the antitumor effect of SeV-mIFN-$\beta$/DCs against MH134 tumors and, inversely, markedly enhanced that against B16F10 melanoma. It is still premature, at present, to draw a conclusion, but we believe a possible contribution of $CD4^+/CD25^{high}$ regulatory T lymphocytes (T-reg) may be involved. Recent progress in knowledge about how tumors escape from host immune surveillance shows an essential contribution of T-reg in tumor immunity (41). The distinct effects of CD4 depletion between two different tumor types, however, may imply that the findings depend on the cell sources, so further study is called for to determine the tumor and/or DC factors contributing to the function of T-reg.

Can SeV/DC show a significant anticancer effect over that seen using current DC immunotherapy in clinical use? It may be premature to suggest this, however, the present study implies some important advantages of SeV/DC-based cancer immunotherapy. In this study, we showed that SeV/DC therapy revealed a strong antitumor effect over that seen with the use of LPS/DCs, which can produce one of the strongest anticancer effects in experimental conditions, and these findings suggest that the therapeutic potential of SeV/DCs warrants further studies, including clinical trials. Because mass production of good manufacturing practice grade SeV is now available, it is not a long way to move SeV/DC-mediated cancer immunotherapy to clinical practice.

In summary, we demonstrated that SeV-modified DCs showed antitumor effects on established tumors in vivo; and we would therefore like to propose a concept for tumor immunotherapy using SeV-modified DCs known as "immunostimulatory virotherapy." The current study results strongly suggest that SeV/DC-based cancer immunotherapy may be an important alternative therapy and that the technique warrants further investigation in research as well as in clinical trials.

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## Disclosures

The authors have no financial conflict of interest.

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# A free radical scavenger but not FGF-2-mediated angiogenic therapy rescues myonephropathic metabolic syndrome in severe hindlimb ischemia

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**Kaneko, Kazuhiro, Yoshikazu Yonemitsu, Takaaki Fujii, Mitsuho Onimaru, Chen-Hao Jin, Makoto Inoue, Mamoru Hasegawa, Toshihiro Onohara, Yoshihiko Maehara, and Katsuo Sueishi.** A free radical scavenger but not FGF-2-mediated angiogenic therapy rescues myonephropathic metabolic syndrome in severe hindlimb ischemia. *Am J Physiol Heart Circ Physiol* 290: H1484–H1492, 2006. First published November 23, 2005; doi:10.1152/ajpheart.01006.2005.—The therapeutic use of angiogenic factors shows promise in the treatment of critical limb ischemia; however, its potential for myonephropathic metabolic syndrome (MNMS), a fatal complication caused by arterial reconstruction, has not been elucidated. The objective of this study was to evaluate the effectiveness of recombinant Sendai virus-mediated gene transfer of fibroblast growth factor-2 (FGF-2) directly compared with that of a radical scavenger, MCI-186, in a rat model of MNMS. MNMS was surgically induced by aortic occlusion below renal arteries for 4 h, followed by 6 h of reperfusion. Administration of MCI-186 (twice; iv 5 min before induced ischemia and ip 5 min before reperfusion; 10 mg/kg, respectively), but not FGF-2 gene transfer (once, 48 h before induced ischemia), dramatically prevented the increase of serum biochemical markers as well as the edema of the gastrocnemius muscle. The effect of MCI-186 was accompanied by the marked suppression of the neutrophilic infiltration into the local (muscle) and remote (lung) organs. Although serum and muscular levels of a neutrophil-chemoattractant (growth-related oncogene/cytokine-induced neutrophil chemoattractant-1) were not affected by any treatment, the serum level of soluble intercellular adhesion molecule-1 was decreased by treatment with MCI-186 but not by treatment with FGF-2. These results suggest the distinct mechanism of MNMS from critical limb ischemia without reperfusion. Therefore, radical scavenging should be paid more attention than therapeutic angiogenesis when arterial circulation is reconstructed.

fibroblast growth factor-2; free radicals; critical limb ischemia; neutrophils

IN THE LAST DECADE, a number of experimental studies have suggested the possible utility of angiogenic growth factors ("therapeutic angiogenesis") to treat limb ischemia, as well as ischemic heart diseases (30, 31, 36). Emerging evidence in clinical trials for limb ischemia, however, has shown a relatively limited outcome of therapeutic angiogenesis, in both protein and gene therapies, in double-blinded placebo-controlled studies (17, 23, 26). Therefore, further effort should be put into the clinical evaluation of the potentials of therapeutic angiogenesis, including the choice of the angiogenic factors, optimized dose and local level of the dose, and indications for clinical stages (4, 5).

In a recent series of experimental studies, we demonstrated that intramuscularly boosted expression of basic fibroblast growth factor (bFGF or FGF-2), which is a prototype polypeptide for angiogenesis, by a highly efficient gene transfer vector, recombinant Sendai virus (SeV) (37), constantly showed efficient therapeutic effects in acute severe hindlimb ischemia of mice (19, 21, 25, 32), as well as chronic limb ischemia of rabbits (25). These data revealed the critical role of endogenous angiogenic factors that are induced by FGF-2, including vascular endothelial growth factor (VEGF) (19, 32) and hepatocyte growth factor (HGF) (21), and more importantly, the data revealed that the effect of FGF-2 was highly dose dependent, requiring >2.5-fold higher local protein level compared with the endogenous expression of it in mice (25).

The indication of angiogenic therapy may possibly be extended from no-choice patients with critical limb ischemia to the adjuvant therapy for individuals with arterial reconstruction, when its efficacy in the clinical setting can be determined. As part of this extension, myonephropathic metabolic syndrome (MNMS) (7) should be an important target of this new therapeutic strategy. MNMS, a lethal disease after 6 h of "golden time" of vascular reconstruction for acute arterial obstruction, is characterized by pentalogy (oliguria, hyperkalemia, metabolic acidosis, myoglobinemia, and azotemia), affecting multiple organ failure, and results in the death of patients at a high rate (7). Because MNMS is a fatal state of ischemia-reperfusion (I/R) injury of the lower extremity (2, 7), boosted expression of FGF-2 may have a beneficial effect because several experimental studies demonstrated the protective property of FGF-2 against I/R injury in the heart (12, 13, 22, 24). Importantly, FGF-2 is known to have a direct protective activity for skeletal muscles undergoing ischemia-induced injury (18), a finding supported by our previous study using a mouse model of acute severe hindlimb ischemia (19).

For these reasons, we here examined whether SeV-mediated FGF-2 gene transfer might rescue the rat model of MNMS, directly compared with the effect of a newly developed and now clinically available drug of a free radical scavenger, MCI-186 (edaravone), which has been shown to attenuate I/R injury in several organs (10, 29). Unexpectedly, we here show that only MCI-186, but not FGF-2, rescued MNMS in the rat model.

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## MATERIALS AND METHODS

*Animals.* Six-week-old male Wistar rats, weighing 200–250 g (KBT Oriental, Charles River grade, Tosu, Saga, Japan), were used in this study. The experimental protocol using animals was submitted to and approved by the Institutional Committees for Animal Experiments and Experiments for Recombinant DNA and Infectious Pathogens at Kyushu University (approval No. 11-76 and 16-94). The animal experiments were done in accordance with recommendations for the proper care and use of laboratory animals, and the law (No. 105) and notification (No. 6) of the Japanese government were also followed.

*Gene transfer agents and MCI-186.* High titer stocks of SeVs expressing human FGF-2 (SeV-hFGF2) and firefly luciferase (SeV-luciferase) used in this study were prepared as described previously (19, 21, 32, 37). Virus titer was determined by hemagglutination assay using chicken red blood cells and was kept at −80°C until use. MCI-186 was a kind gift from Mitsubishi Pharma.

*Experimental protocol.* The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). The surgical procedure for I/R was performed as described in Fig. 1*A*. Briefly, ischemia was induced by occluding the abdominal aorta under the renal artery and bilateral femoral arteries by 5-0 nylon for 4 h. Reperfusion was then done by removing the nylon threads. The experimental groups were designed as shown in Fig. 1*B*. For *group 3*, 0.5 ml of vector solutions, containing $1 \times 10^8$ plaque-forming units of SeV-hFGF2, was injected into five portions of each lower limb (3 for the thigh and 2 for the calf) intramuscularly 48 h before surgery-induced ischemia. For *group 4*, 0.5 ml of MCI-186 solution (10 mg/kg in PBS) was administered intravenously 5 min before induced ischemia and intraperitoneally 5 min before reperfusion. For *group 2*, as a control of *group 4*, the same volume of PBS was administered instead of MCI-186. Six hours after reperfusion, all animals were killed by an overdose of anesthetic, and materials were subjected to the following evaluations. *Group 1* consisted of untreated animals. Three independent experiments (*tracks 1–3*) were done to obtain different materials from rats, as indicated in Fig. 1*C*, including two animals that received SeV-luciferase as a control for *group 3* in *tracks 1* and *2*, showing similar results to those seen in *group 2* (data not shown). Therefore, a total of 68 animals were used in this study, and no animal died in the series of experiments.

*Biochemical analysis.* Serum levels of blood urea nitrogen (BUN), creatinine (Cr), potassium (K), creatine phosphokinase (CPK), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and aldo-

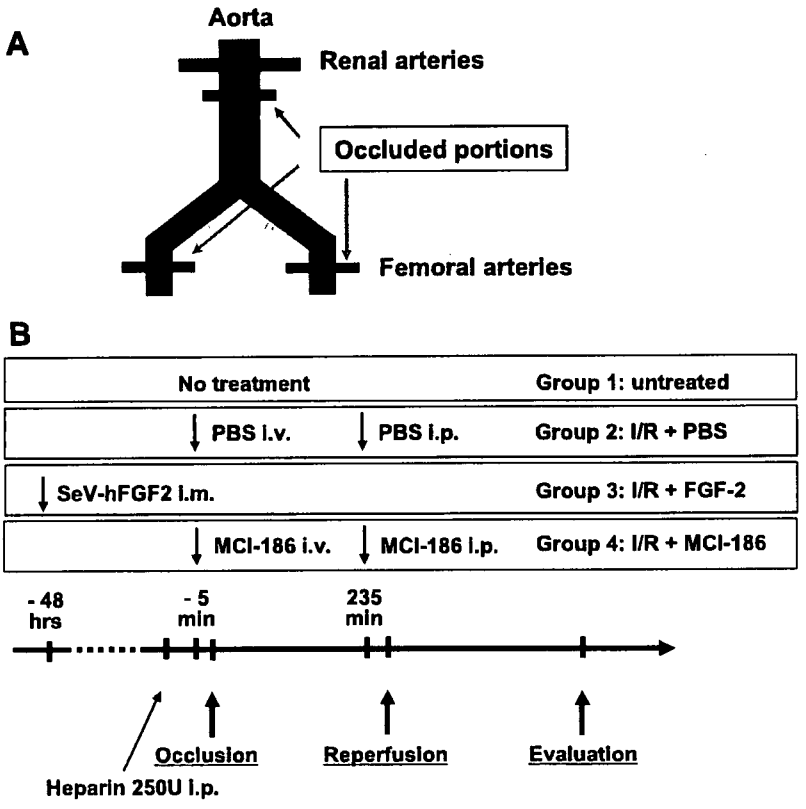


Fig. 1. Experimental procedure and protocol. *A*: scheme of occluded aorta/arteries for the surgically induced rat model of myonephropathic metabolic syndrome (MNMS). *B*: treatment regimen and experimental groups. For *group 3*, Sendai virus expressing human fibroblast growth factor-2 (SeV-hFGF2) was injected into the limb muscles so that the FGF-2 would show peak expression at the arterial occlusion. I/R, ischemia-reperfusion. *C*: experimental tracks for evaluation. The same experiment as indicated in *B* was performed in each track. HE, hematoxylin and eosin; GRO/CINC-1, growth-related oncogene/cytokine-induced neutrophil chemoattractant-1; sICAM-1, soluble intercellular adhesion molecule-1; WBC, white blood cell.

**C**

**Track 1. (n=6/group, total=24 animals)**

**Rt. Gastrocnemius muscle: ELISA for FGF-2 and GRO/CINC-1**

**Track 2. (n=6/group, total=24 animals)**

**Rt. Gastrocnemius muscle: H.E. and immunohistochemistry**
**Lt. Gastrocnemius muscle: wet/dry weight ratio**
**Rt. Lung: H.E. and immunohistochemistry**
**Serum: Serum chemistry and ELISA for GRO/CINC-1 and sICAM-1**

**Track 3. (n=4/group, total=16 animals)**

**Whole blood: WBC count and neutrophil count**

lase were measured in serum samples taken from the abdominal aorta when all animals were killed at *track 2*. Concentrations of BUN, Cr, K, CPK, AST, LDH, and aldolase were determined by an automatic analyzer (Research Testing Department, SRL, Hachiohji-shi, Tokyo).

*Wet-to-dry weight ratio.* The left gastrocnemius muscle was excised from animals at *track 2*, and the wet weight was determined. The muscle was dried at 60°C in a convection oven for 72 h, and then the dry weight was measured. The wet-to-dry weight ratio was calculated as an indicator of muscle edema (11).

*Immunohistochemistry.* Infiltrated neutrophils within lower limb muscle and lung were identified by immunohistochemistry. Right gastrocnemius muscle (at 10 mm below the knee) and right lung were excised from animals at *track 2* and fixed in 10% buffered formalin. Each section was embedded in paraffin, and 2-μm-thick sections were stained with hematoxylin and eosin; additionally, the serial sections were subjected to immunohistochemical studies. Rabbit anti-myeloperoxidase (anti-MPO) (Dako, Glostrup, Denmark, 1:200 in PBS) (1, 38) was used for a primary antibody. Immunohistochemical examinations were performed as described previously (20). In brief, deparaffinized sections were incubated with 3% nonfat milk, and the reaction with peroxidase-labeled secondary antibody (Envision System, Dako) followed reaction of the primary antibody. Positive reaction was visualized with 3,3′-diaminobenzidine tetrahyrochloride to give the reaction product a brown color, and then the sections were counterstained with hematoxylin. Nonimmune rabbit IgG was also used instead of the respective primary antibody as each negative control. MPO-positive cells in randomly selected 50 foci of high-powered view (×200) per slide were counted under a light microscope, and the mean value was used as the MPO-positive cells/focus in each section.

*ELISA.* Serum and/or muscular levels of FGF-2, growth-related oncogene/cytokine-induced neutrophil chemoattractant-1 (GRO/CINC-1) (a rodent homolog of human IL-8) (33, 34), and soluble intercellular adhesion molecule-1 (sICAM-1) were determined by ELISA. Commercially available assay systems were used [R&D Systems (Minneapolis, MN) for FGF-2 and sICAM-1, and IBL (Gunma, Japan) for GRO/CINC-1], according to the manufacturers' instructions. Quantikine system for FGF-2 is available for human, murine, rabbit, rat, and monkey FGF-2 (19, 21, 25, 32). For muscle preparation, the right gastrocnemius muscle was homogenized in lysis buffer with protease inhibitor cocktail (19, 21, 25, 32), the insoluble material was removed by centrifugation at 12,000 rpm for 10 min, and the supernatant was subjected to ELISA. Serum samples were used for ELISA after appropriate dilutions. All samples were stored at −20°C and analyzed within 24 h after collection.

*White blood cell count and white blood cell differential count for neutrophils.* The white blood cell (WBC) count was determined with an automatic hemocytometer using whole blood taken from the abdominal aorta when all animals were killed at *track 3*. The neutrophil count was determined from a smear of each sample with Giemsa staining.

*Statistical analysis.* Data were presented as means ± SE. All statistical comparisons were performed using one-way ANOVA with Fisher protected least significant difference test for multiple comparisons. A probability value of $P < 0.05$ was considered significant.

## RESULTS

*Local concentration of FGF-2.* Using muscular tissue at *track 1*, we determined the local level of FGF-2 to investigate the possible alteration of FGF-2 expression due to the intervention and the level of SeV-mediated hFGF-2 gene transfer.

As shown in Fig. 2*A*, all animals except for those in *group 3* (FGF-2) showed no significant increase of local FGF-2 level, indicating that the surgical treatment for I/R did not affect FGF-2 expression. The protein level of FGF-2 in *group 3* reached >2.5-fold greater than that seen in untreated animals (*group 1*), a level that showed a significant hindlimb-salvaging effect of FGF-2 gene transfer in a murine severe limb ischemia model, as previously demonstrated (25).

*The free radical scavenger MCI-186, but not FGF-2, inhibits muscular and renal damage.* Before the start of the main experiments shown in Fig. 1, *B* and *C*, we determined the effective dose and the timing of MCI-186 administration in use of relatively small numbers of animals ($n$ = 3/each group) by assessing serum CPK, AST, LDH, and aldolase as an initial study (Fig. 2*B*). We here used two independent doses (3 vs. 10 mg/kg) with dual injection and 10 mg/kg at single (ip 5 min before reperfusion) vs. dual (iv 5 min before induced ischemia and ip 5 min before reperfusion) injection. As a result, 10 mg/kg with dual administration protocol showed optimized effect of MCI-186 on the serum level of CPK and aldolase (Fig. 2*B*), similar to AST and LDH (data not shown); therefore, we chose this regimen in the following experiments.

With the use of serum samples obtained in animals at *track 2*, biomarkers indicating the muscular damage (CPK, AST, aldolase, and LDH) and renal function (BUN, K, and Cr) were determined (Fig. 2, *C* and *D*).

Serum concentrations of CPK, AST, aldolase (Fig. 2*C*), LDH (data not shown), BUN, and K (Fig. 2*D*) were significantly increased in the animals of the I/R and SeV-hFGF2 groups, and the increase of these markers was completely abolished in the MCI-186 group. There was no significant difference in the Cr concentration among the groups, but Cr concentration showed a similar tendency to that of the other parameters (data not shown). Additionally, we performed the same experiment with 6 h of ischemia before reperfusion as a preliminary study; however, >70% of animals died within the observation period (data not shown), suggesting that ischemia longer than 4 h might induce injury too severe for experimental evaluation.

These results thus indicate that the current animal model should be a relevant rat model of MNMS inducing extensive muscular and renal damage.

*MCI-186, but not FGF-2, inhibits muscular edema.* Next, we evaluated the edema of muscular tissue at *track 2* because tissue edema has been reported as an important indicator of I/R injury (11).

As shown in Fig. 3*A*, histopathological sections showed apparent interstitial edema in the I/R and I/R + FGF-2 groups, and the effect was largely diminished by MCI-186 treatment. Furthermore, the weight-to-dry weight ratio was significantly increased in the animals of the I/R and SeV-hFGF2 groups, and this effect was almost completely inhibited by MCI-186 (Fig. 3*B*).

*MCI-186, but not FGF-2, inhibits neutrophilic infiltration to muscles and lungs.* We next examined the neutrophilic infiltration into the local (right gastrocnemius muscle) and distant (right lung) organs by immunohistochemical labeling of MPO using samples obtained at *track 2* because neutrophils have been suggested to play a significant role in the progression of I/R injury (1, 38).

As shown in Fig. 4, *A* and *B*, I/R caused marked infiltration of neutrophils in both muscles and lung, but neither was affected by FGF-2 gene transfer. In contrast, the number of MPO-positive neutrophils was dramatically diminished by MCI-186 treatment in both organs.

*MCI-186, but not FGF-2, inhibits serum sICAM-1 level without a significant effect on a neutrophil chemoattractant.* To seek the possible mechanism of the preventive effect of MCI-

186 in the rat model of MNMS, we focused on the neutrophils and their infiltration pathway because local accumulation of neutrophils has been shown to play a significant role in the progression of I/R injury (3).

We first examined the number of neutrophils in the blood by using *track 3* animals. As shown in Fig. 5*A*, a dramatic decrease in the whole WBC number was found in *groups 2–4*, suggesting the absorption due to the recruitment of WBCs into

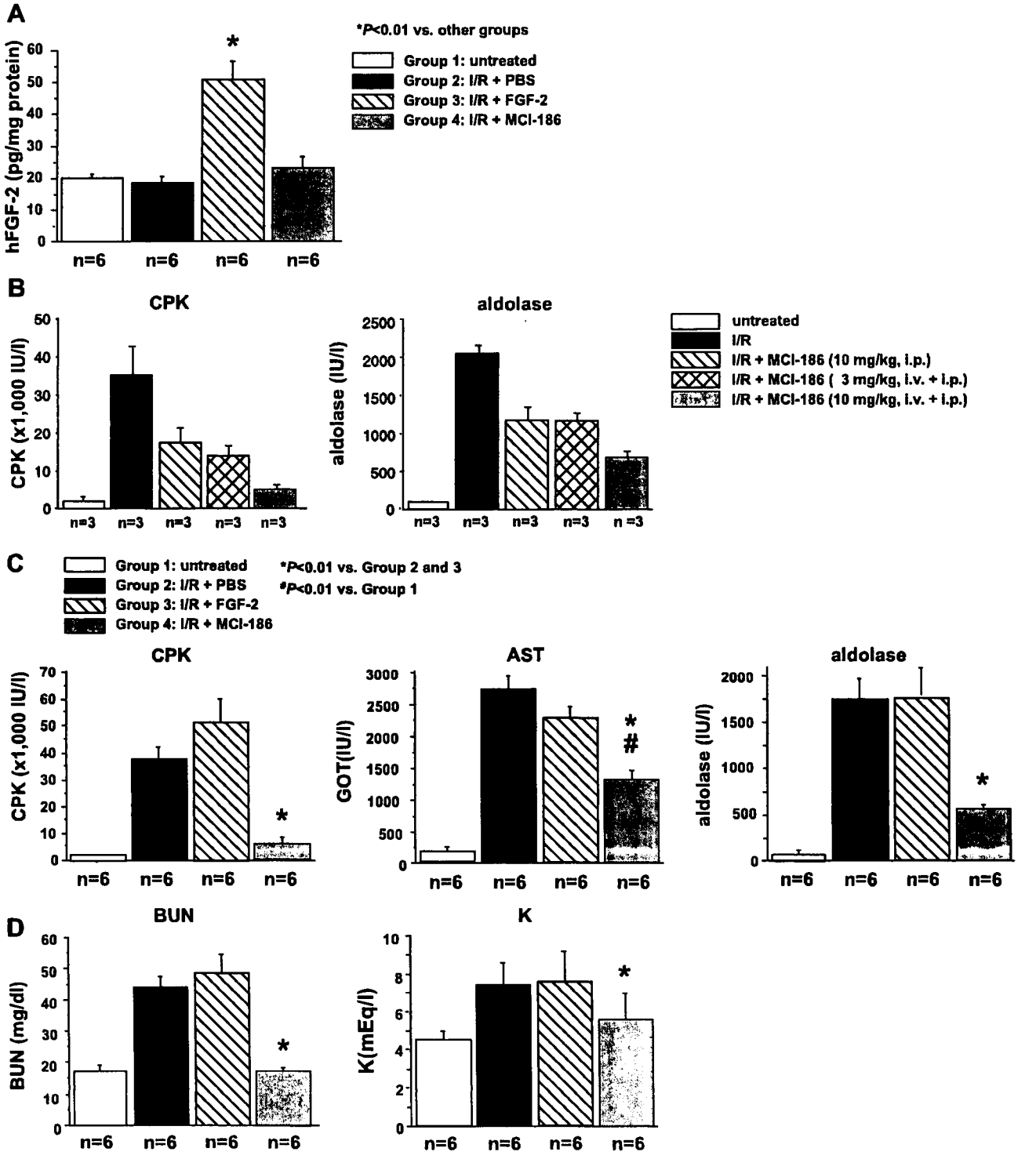


Fig. 2. Expression of intramuscular FGF-2 by ELISA (*A*), determination of the timing and dose of MCI-186 (*B*), and serum levels of biomarkers for muscular (*C*) and renal (*D*) damage. *A*: intramuscular level of FGF-2. The right gastrocnemius muscular samples in *track 1* were obtained 6 h after reperfusion and subjected to an ELISA that recognizes both murine and hFGF-2. *$P < 0.01$ vs. other groups. *B*: initial optimization study assessing the timing and dose of MCI-186. Dual administration of MCI-186 at 10 mg/kg showed optimized effect on the serum level of creatine phosphokinase (CPK) and aldolase. *C*: serum levels of biomarkers for muscular damage in *track 2* animals. AST, aspartate aminotransferase; GUT, glutamic-oxaloacetic transaminase. *$P < 0.01$ vs. *groups 2* and *3*; #$P < 0.01$ vs. *group 1*. *D*: serum levels of biomarkers for renal damage in *track 2* animals. BUN, blood urea nitrogen. *$P < 0.01$ vs. *groups 2* and *3*.

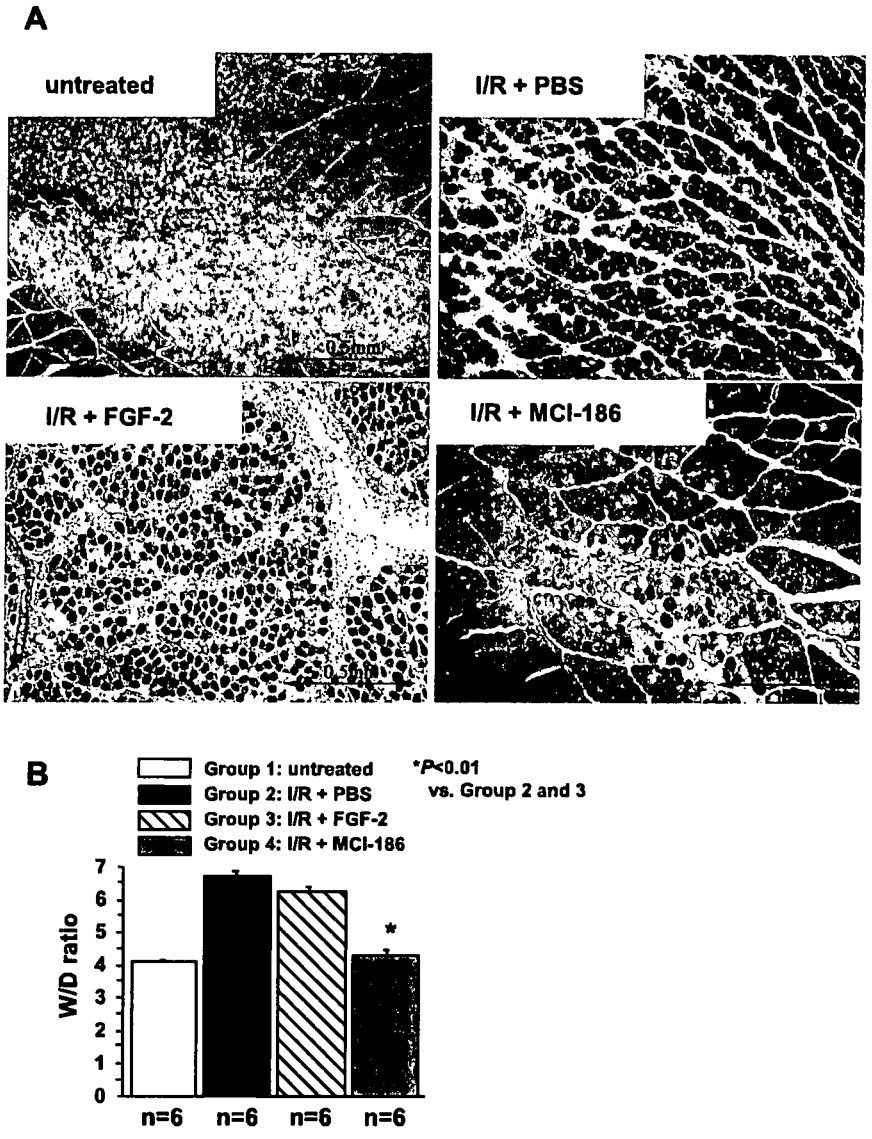


Fig. 3. Histological (*A*) and quantitative (B) examination of muscular edema. The left gastrocnemius muscular samples in *track 2* were obtained 6 h after reperfusion. *A*: typical and representative histopathological findings of muscles. Note that interstitial edema is evident in group 2 (I/R + PBS) and *group 3* (I/R + FGF-2), a finding that is markedly inhibited in *group 4* (I/R + MCI-186). Bar, 0.5 mm. *B*: bar graph indicating wet-to-dry weight (W/D) ratio. $*P < 0.01$ vs. *groups 2* and *3*.

the reperfused and distant organs. However, the number of circulating neutrophils was not significantly different among groups tested (Fig. 5*A*, *right*).

Next, we measured the serum level of a neutrophil chemoattractant that is a rodent homolog of human IL-8, GRO/CINC-1, by using samples from *track 2* animals. As shown in Fig. 5*B*, I/R strongly induced a rise in the serum level of GRO/CINC-1, which was not affected by any other treatment tested. Similar results were observed in the local protein content of GRO/CINC-1 in muscles from *track 1* animals (Fig. 5*B*, *right*).

The above results taken together suggested that the preventive effect of MCI-186 did not include the proliferative and chemotactic processes of neutrophils. Therefore, we next assessed the expression of ICAM-1, which has been shown to be a major adhesion molecule for neutrophilic infiltration. We here examined the soluble form of ICAM-1 (sICAM-1) in the serum, because the serum level of sICAM-1 has been shown to reflect the expression of endothelial and membrane-bound ICAM-1 (16) and to be an important biomarker for endothelial function and inflammatory context (8, 9).

As shown in Fig. 5*C*, I/R significantly increased the serum level of sICAM-1, which was not affected by FGF-2 gene transfer. In contrast, treatment with MCI-186 significantly downregulated sICAM-1 at the control level, suggesting that the preventive effect of a free radical scavenger, MCI-186, to neutrophil infiltration might involve the suppression of the endothelial expression of ICAM-1.

## DISCUSSION

Recent experimental studies have suggested the possible utility of angiogenic factors, including FGF-2, not only in the treatment of "tissue ischemia" (19, 21, 25, 32) but also "ischemia-reperfusion-mediated tissue injury" (12, 13, 24). We here demonstrated, however, the stronger protective effect of a free radical scavenger to the rat model of MNMS compared with that by FGF-2 gene transfer. These findings suggest the distinct molecular mechanism between tissue ischemia and I/R injury, implying that the scavenging of free radicals should be paid more attention than the restoration of blood perfusion when arterial reconstruction must be performed in the clinical setting.

An important advance obtained in this study is to demonstrate the information regarding the direct comparison of the effects of angiogenic therapy and the scavenging of radicals,

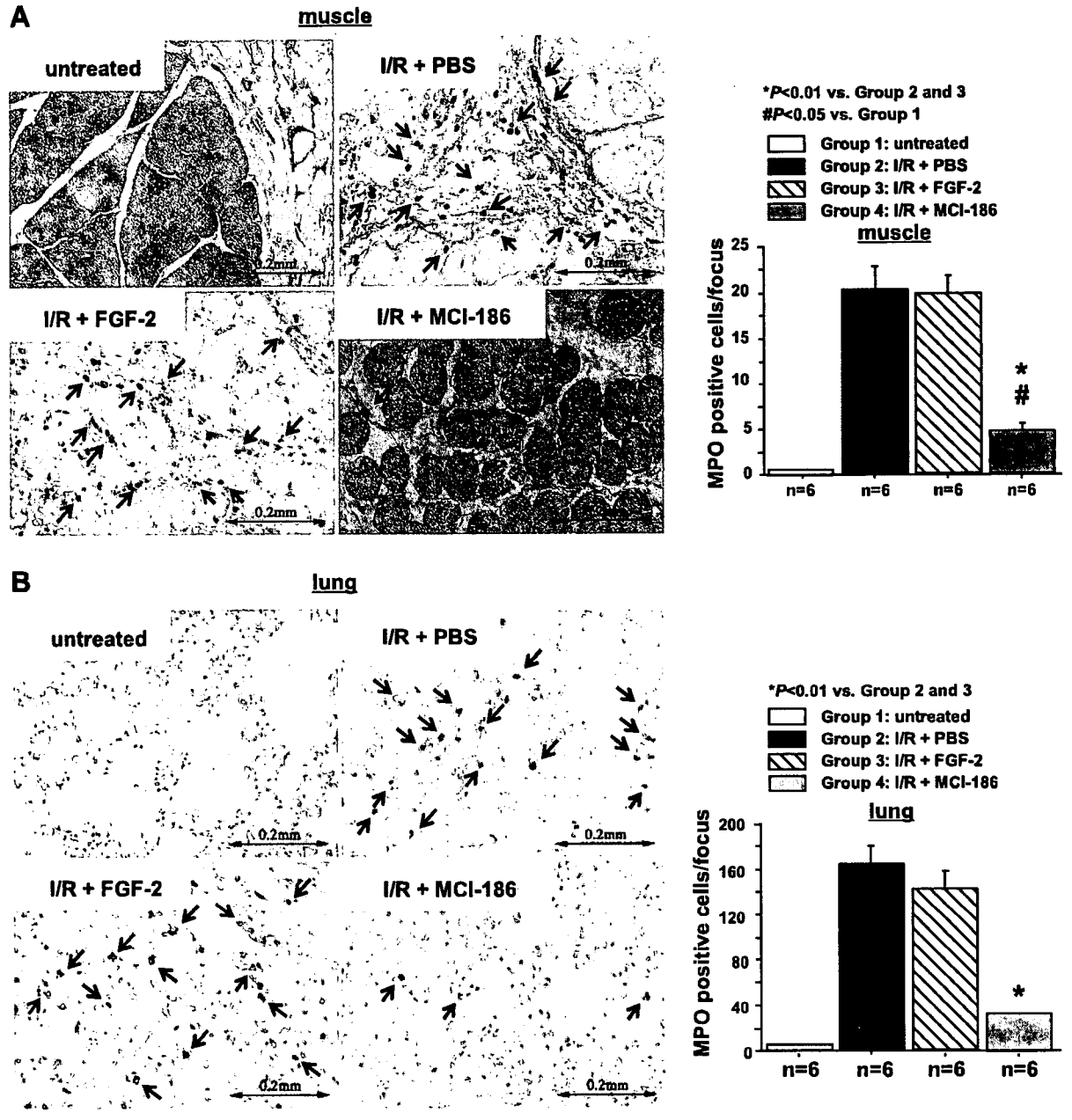


Fig. 4. Immunohistochemistry for myeloperoxidase (MPO) identifying neutrophilic infiltration into the local (muscle, *A*) and distant (lung, *B*) organs. Randomly selected 50 foci of high-powered view (×200) per slide were counted under a light microscope, and the mean value was used as the MPO-positive cells/focus in each section. *$P < 0.01$ vs. *groups 2* and *3*; #$P < 0.05$ vs. *group 1*. *A*: MPO-positive neutrophils infiltration (arrows) into the local organ (muscle). A marked increase in the number of MPO-positive cells is evident in *group 2* (I/R + PBS) and *group 3* (I/R + FGF-2), a finding that is dramatically inhibited in *group 4* (I/R + MCI-186). *B*: similar results to *A* in the local organ (muscle) were obtained in the distant organ (lung).

both of which were shown as effective, in a relevant rat model for MNMS. There is, however, still an unsolved issue to be clarified regarding the molecular and cellular mechanisms of free radicals in the progression of MNMS. We here attempted to address this issue in the current study.

One important question is what cell species are major targets of free radicals in MNMS. Because muscular edema has been shown as a result of microvascular permeability induced by vascular endothelial cell damage due to free radicals (14), vascular endothelial cells (ECs) would possibly be a critical determinant of free radical-induced tissue injury. This also suggests that MCI-186 may target ECs in the model used in the present study, and this is supported by the current results indicating the suppression of ICAM-1 expression that is specifically induced by ECs and, inversely, the lack of any effect on the expression of GRO/CINC-1 that is expressed by other cell types, including monocyte/macrophages (27). This is well supported by an in vitro experiment indicating the preventive effect of MCI-186 on vascular EC injury (35), so it is reasonable to conclude that vascular EC injury due to free radicals is the major cause of MNMS and that MCI-186 protects ECs from radical species.

The second question is whether the inhibition of neutrophil infiltration into the local and remote organs may be a cause or

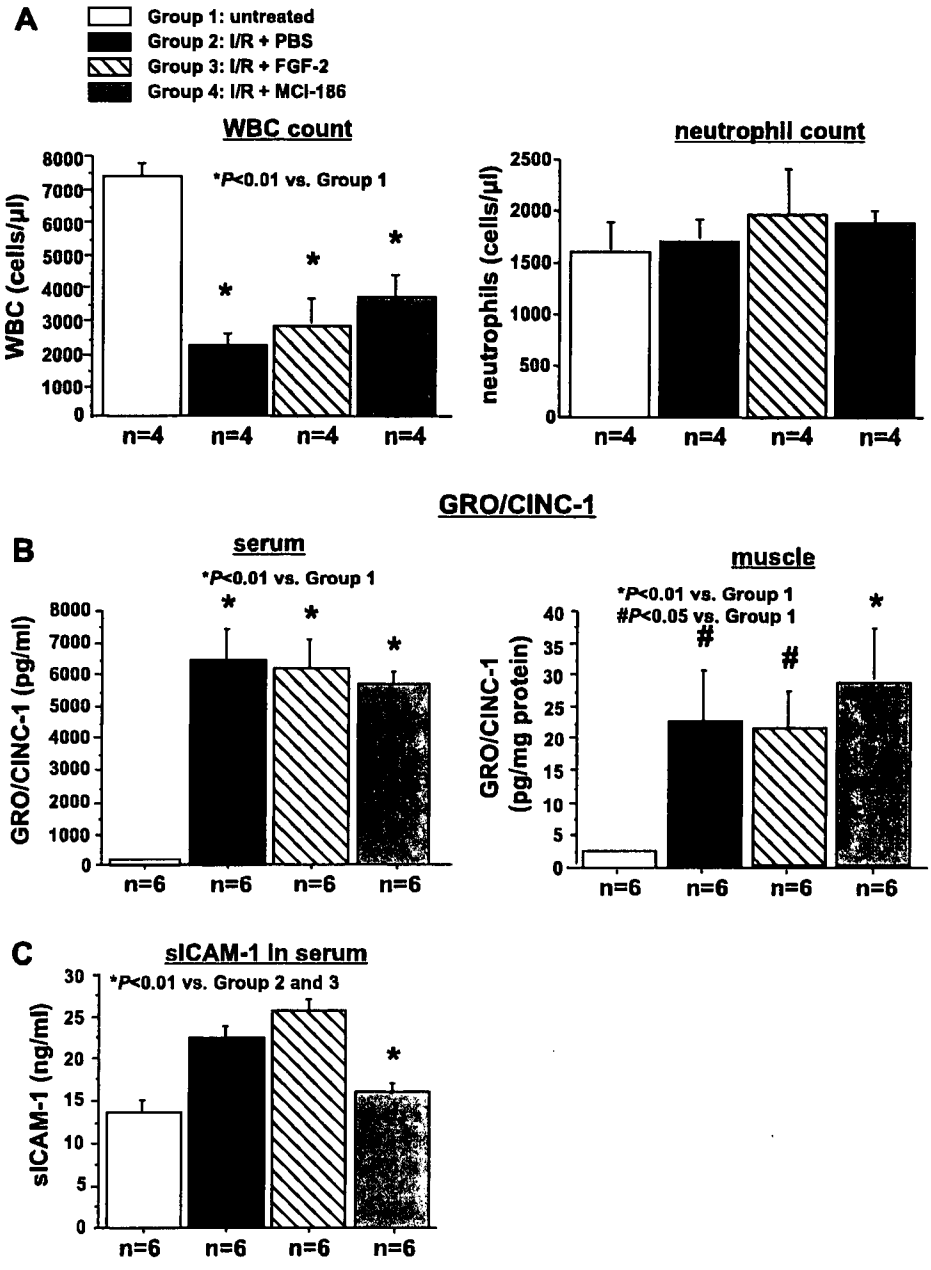


Fig. 5. Assessment of neutrophic infiltration-related parameters. *A*: WBC count (*left*) and the number of neutrophils (right) in the whole blood of animals at *track 3*. Possible absorption of WBCs due to I/R injury is seen in all groups (*groups 2–4*) except for the untreated controls (*group 1*). *$P < 0.01$ vs. *group 1*. *B*: protein levels of a typical neutrophil chemoattractant, GRO/CINC-1 (a rodent homolog of human IL-8), in serum (*left*; *track 2* animals) and muscles (*right*; *track 1* animals) assessed by ELISA. I/R strongly induced GRO/CINC-1 in both serum and muscle, which was affected by neither FGF-2 nor MCI-186. *$P < 0.01$ vs. *group 1*; #$P < 0.05$ vs. *group 1*. *C*: serum levels of the soluble form of a typical neutrophilic adhesion molecule (sICAM-1) in *track 2* animals assessed by ELISA. I/R accelerated the expression of sICAM-1, which was significantly inhibited by MCI-186 (*group 4*) but not by FGF-2 (*group 3*). *$P < 0.01$ vs. *groups 2* and *3*.

a result of multiple organ failure in MNMS and of EC protection by MCI-186, as indicated by the reduced expression of sICAM-1. As shown in an important study using a liver injury model, a deficiency of ICAM-1 resulted in reduced liver injury associated with a decreased neutrophilic infiltration (6), suggesting that recruitment of neutrophils into the inflammatory foci via ICAM-1 is the major cause of tissue injury. From this point of view, anti-ICAM-1 therapy, including EC protection by MCI-186, might be an important strategy for the rescue of MNMS via reducing neutrophil-EC interactions.

The biological role of the increase of sICAM-1 in inflammatory diseases has not been well understood. As indicated in a number of studies, serum sICAM-1 level has been shown to correlate well with the EC expression of membrane-bound ICAM-1 (8, 9, 16), while sICAM-1 functions as a decoy for leukocyte adhesion inhibiting leukocyte-EC interaction (15, 28). Our preliminary study could not show the direct inhibitory effect of MCI-186 on ICAM-1 as well as sICAM-1 expression of human umbilical ECs stimulated by LPS and TNF-α in vitro, suggesting that the reduction of sICAM-1 in vivo has only an indirect effect on ECs. It has been demonstrated that membrane-bound ICAM-1 could be cleaved by metalloproteases, and, therefore, the reduction of the protease activity of such molecules should be involved in the effect of MCI-186. Further studies are called for to clarify this point further.

Considering a clinical setting, we have performed some additional experiments with later administration of MCI-186 after reperfusion in the present rat model of MNMS; however, very little effect was shown (data not shown), indicating that pretreatment of a free radical scavenger would be absolutely necessary to obtain a sufficient preventive effect on I/R injury in lower limb ischemia. Therefore, this drug should be administered, at least, just before reperfusion.

We here demonstrated that FGF-2 gene transfer was not effective and not toxic to the acute phase (6 h after reperfusion) after I/R injury of severe hindlimb ischemia; however, its benefit during the chronic phase has not yet been evaluated. The distinct mechanism between a free radical scavenger and FGF-2 gene transfer on limb ischemia may possibly show the additive effect on the chronic phase of the disease. Because such information is important in the clinical setting, we are now assessing this as a subsequent experiment.

In summary, we here demonstrated that free radicals played a critical role in the rat model of MNMS, a distinct mechanism from hindlimb ischemia. The beneficial effect of a free radical scavenger, but not FGF-2 therapy, was due to the inhibition of neutrophilic infiltration within local and remote organs, probably via inhibiting expression of sICAM-1. Therefore, the scavenging of free radicals generated after reperfusion should be paid more attention than angiogenesis when arterial circulation is reconstructed.

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