Vinorelbine is Effective for the Malignant Pleural Effusion Associated with Lung Cancer in Mice

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Abstract. *Malignant pleural effusion (MPE) is associated with advanced-stage lung cancer. Vinorelbine (VNR) is a semisynthetic vinca alkaloid with strong antitumor activity in non-small cell lung cancer (NSCLC). The purpose of this study was to evaluate the effect of VNR on the production of MPE associated with lung cancer. To investigate the therapeutic efficacy of VNR in the production of MPE in mice, cells of the murine lung cancer cell line, 3LL, were injected into the pleural space of mice, and VNR was administered once intravenously. Treatment with VNR almost completely inhibited tumor growth and MPE production. In addition, immunohistochemical staining revealed that neovascularization and vascular endothelial growth factor (VEGF) expression were markedly decreased in VNR-treated tumors compared with those of the control tumors. Treatment of 3LL cells with high concentrations of VNR (5 or 10 nM) significantly inhibited cell proliferation in vitro. Interestingly, low concentrations of VNR (1 or 2.5 nM) did not affect 3LL cell proliferation, but markedly reduced VEGF expression in these cells. These results suggest that VNR may be effective for the treatment of MPE associated with lung cancer not only by its cytotoxic effect but also through down-regulation of VEGF expression in lung cancer cells.*

Malignant pleural effusion (MPE) is a common and distressing condition that is associated with advanced-stage lung cancer. Most patients with MPE experience progressive dyspnea or cough that compromises their daily quality of life (QOL) (1). The appearance of MPE has been reported to indicate a poor prognosis in patients with advanced lung cancer, being associated with high morbidity and mortality (2, 3). Previous reports have revealed that tube drainage followed by induction of pleural sclerosis with instillation of antibiotics or antineoplastics are useful for controlling PE and improving the QOL of patients (4, 5). Recently, Yoshida K *et al*. have reported on a randomized phase II trial for the management of MPE in non-small cell lung cancer (NSCLC). They indicated that intrapleural instillation of bleomycin, cisplatin plus etoposide, or OK-432 (a pulverized product of heat-killed *Streptococcus pyogenes*) is effective for the management of MPE in previously untreated NSCLC (6). However, the efficacy of these treatments is variable and does not extend the survival of patients with lung cancer. Furthermore, we occasionally encounter NSCLC patients with MPE who require treatment but cannot undergo tube drainage and pleurodesis procedures. For such patients, systemic chemotherapy is selected for treatment to reduce MPE. Presently, there are only a limited number of studies describing which chemotherapeutic agents are effective for management of MPE associated with lung cancer. Therefore, there is a need to further identify anticancer agents that reduce MPE associated with lung cancer.

Vinorelbine (VNR) is a semisynthetic vinca alkaloid derivative that demonstrates strong antitumor activity in various malignancies (7). It exerts a cytotoxic effect upon rapidly proliferating tumors through prevention of mitotic spindle formation by inhibiting the polymerization of tubulin into microtubules (8, 9). Animal studies have shown that VNR has favorable antitumor activity on lung cancer cells and can induce cellular differentiation (10). Recently, much interest has been focused on the therapeutic efficacy of VNR in NSCLC. Clinical studies demonstrated that VNR has antitumor activity in patients with advanced NSCLC as a single agent and in combination with other anticancer agents including cisplatin or gemcitabine (11-13). Moreover, VNR

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has been proven to improve the patient's QOL as well as prolong survival of elderly NSCLC patients with poor performance status (14). VNR would appear to be a good candidate as an anticancer agent for NSCLC patients with MPE. However, the therapeutic efficacy of VNR on MPE of lung cancer has not yet been well elucidated.

In this study, we developed a mouse MPE model using 3LL cells, which is similar to the Stathopoulos's model, where they share common cellular and biochemical features of the human MPEs (15), and investigated whether VNR is effective for MPE associated with lung cancer.

Materials and Methods

Cell line and reagents. The murine lung cancer cell line, 3LL, was purchased from the American Type Culture Collection (Manassas, VA, USA), and maintained in RPMI-1640 medium (Koujin Bio, Saitama, Japan) containing 10% (v/v) fetal calf serum (FCS). The monoclonal antibody against murine CD31 was purchased from Pharmingen (San Diego, CA, USA). The polyclonal antibody p-20 against epitopes of murine vascular endothelial growth factor (VEGF) was purchased from Santa Cruz Biotechnology (CA, USA). The recombinant human VEGF165 (rhVEGF165) was purchased from R&D Systems (Minneapolis, MN, USA).

In vitro cell proliferation assay. One thousand 3LL cells were seeded in 96-well culture plates and cultured with RPMI-1640 medium containing 10% (v/v) FCS. After 24 h incubation, the complete mediums were replaced with 2% FCS in RPMI-1640 medium, and VNR (1, 2.5, 5 or 10 nM) was added to the cells, followed by their incubation for an additional 48 h at 37˚C with 5% CO_2. After incubation, the Cell Counting Kit 8 (Dojindo, Kumamoto, Japan) was added to each well to obtain a final volume ratio of 1/10 (v/v). The plate was further incubated at 37˚C for 4 h until color development. Absorbance was determined at 450 nm on a microplate reader with microplate manager (Bio-Rad, Richmond, CA, USA). All experiments were carried out in triplicate.

Sandwich enzyme-linked immunosorbent assay (ELISA). A total of 2.5×10^5 3LL cells were cultured in 6-well plates with 10% FCS in RPMI-1640 medium overnight. After overnight culture, the medium was replaced with 2% FCS in RPMI-1640 medium, VNR (1 or 2.5 nM) was added to the cells and they were cultured at 37˚C for 48 h. Secreted murine VEGF protein in the culture supernatant was determined with a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Quantitative real-time PCR. Total RNAs were extracted from control and VNR-treated 3LL cells after 24 h treatment with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized from 2 μg of total RNA of each cell sample using a Gene Amp RNA PCR Kit (Applied Biosystems, Branchburg, NJ, USA) according to the manufacturer's instructions. The cDNAs were then used as templates for individual PCR reactions using specific TaqMan Gene Expression Assays (Roche, Branchburg, NJ, USA). PCR reactions were carried out using TaqMan Universal PCR Master Mix (Roche). Quantitative PCR analysis was performed using the Applied Biosystem 7500 (Applied Biosystems, CA, USA). GAPDH was used for normalization of expression data.

Mice. Male C57BL/6 mice, 6-7 weeks old, were purchased from Charles River Co., Ltd. (Tokyo, Japan). Male athymic BALB/c nude mice, 6-7 weeks old, were purchased from CREA Japan (Tokyo, Japan). Mice were maintained in our animal facilities under specific pathogen-free conditions. All animal experiments were performed according to the Guidelines on Animal Experimentation as established by Juntendo University, School of Medicine, Dunkyo-Ku, Tokyo, Japan.

Animal studies. For intrapleural injection, C57BL/6 mice were anesthetized by intramuscular injection with 0.1 ml of a mixture of ketamine and xylazine (50 mg/ml of ketamine sodium chloride (Wako Pure Chemical Industries, Osaka, Japan) solution dissolved with saline (Otsuka Pharmaceutical Factory, Tokyo, Japan): Celactal® 2% solution for injection (Bayer Healthcare, Osaka, Japan): saline, 5:1:10). The skin overlying the lateral chest wall was shaved and disinfected, and a 2 to 3 mm-long transverse skin incision was made on the light infrascapular region. Ten thousand 3LL cells suspended in 100 μl RPMI-1640 medium were injected into the pleural cavity through the intercostal space under direct observation. The mice were observed until complete recovery and this procedure was not associated with mortality or morbidity. To investigate whether VNR affects tumor progression in the mouse MPE model, VNR or saline was administered to the mice injected with 3LL cells (n=12 for VNR group, n=20 for saline group). Briefly, VNR (30 mg/kg) or saline was administered once intravenously at day 8 after injection with 3LL cells. After day 13, mice were euthanized by diethylether. The abdominal wall was cut down, and the viscera were retracted to visualize the diaphragm. PE was collected using a 1-ml syringe and the volume of PE was determined with the syringe. Thereafter, the chest wall was cut carefully with scissors and the intrapleural tumors were harvested. The tumors were washed with 0.9% saline and the weights of the tumors were determined with an automatic balance.

The survival study was conducted using mice treated with VNR or saline as described above (n=6 for VNR group, n=6 for saline group). The percentage of increase in life span (% ILS) is defined as follows: % ILS= [(mean survival time of treated/mean survival time of control)-1] ×100.

Immunohistochemical staining. Histological sections were obtained from control and VNR-treated 3LL tumor tissues resected from mice. After resection, tumor tissues were immediately embedded and frozen in Tissue-Tek OCD compound (Miles Laboratories, Elkhart, TN, USA), and sections were cut at 4 μm thickness. Immunohistochemical staining for murine VEGF and CD31 was performed as described elsewhere (16). Briefly, the sections were fixed with 4% paraformaldehyde (PFA) and then incubated at 4˚C overnight with anti-mouse VEGF or anti-CD31 antibody. Specific bindings were detected through avidin-biotin peroxidase complex formation with biotin-conjugated rabbit anti-goat IgG (Dakocytomation, Denmark) for anti-mouse VEGF, or biotin-labeled goat anti-rat IgG (Cedarlane, Ontario, Canada) for anti-CD31 antibody and diaminobendizine (DAB) (Sigma, St. Louis, MI, USA) as the substrate. Staining was absent when isotype-matched immunoglobulin was used as the control.

Vascular permeability assay (Miles assay). To evaluate the effect of VNR on VEGF-induced vascular permeability, the Miles permeability assay was adapted for use with mouse skin. VNR was intravenously administered into BALB/c nude mice once at a dose of 10 or 20 mg/kg.

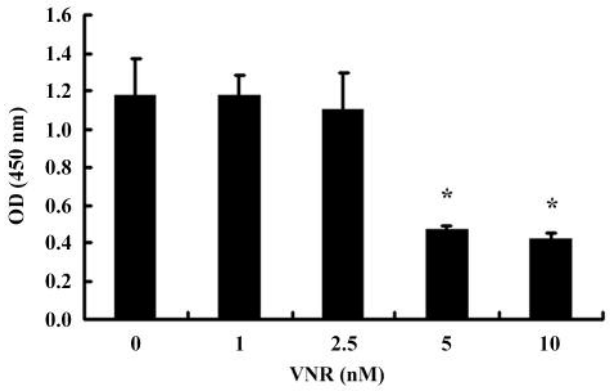

Figure 1. *Effect of VNR on 3LL cell proliferation. 3LL cells were treated with VNR (1, 2.5, 5 or 10 nM) for 48 h. Cell proliferation was assessed with a colorimetric cell counting kit, as measured by absorbance (OD). Data are presented as mean ± SD. *p<0.05 versus 0 nM VNR.*

Two hours after the last administration, nude mice were injected with 0.5% Evans blue dye (200 μl). Ten minutes later, 50 μl of PBS or rhVEGF165 (30 ng/ml) was injected intradermally in rows on the dosal skin. Thirty minutes after the injection with PBS or rhVEGF165, the mice were sacrificed and the skin was excised. Wheals (8 mm in diameter) were resected and incubated in 500 μl of formamide at 37˚C for 48 hours to extract the Evans blue dye. The absorbance of the extracts was read at 630 nm on a microplate reader with microplate manager (Molecular Devices, Sunnyvale, CA, USA). Each study group consisted of six mice.

Statistics. Statistical analysis was performed with analysis of variance (ANOVA). All data are presented as mean±standard deviation. Differences between means were considered statistically significant at $p<0.05$. Differences in survival rates were analyzed with the χ^2 test. Survival curves were compared with the log-rank test.

Results

VNR inhibits 3LL cell proliferation. As shown in Figure 1, high concentrations of VNR (5, 10 nM) significantly inhibited 3LL cell proliferation *in vitro*. Note that low concentrations of VNR (1, 2.5 nM) did not affect *in vitro* 3LL cell proliferation.

VNR reduces VEGF expression of 3LL cells. To verify whether low concentrations of VNR (1, 2.5 nM), which did not inhibit 3LL cell proliferation, affected VEGF secretion from 3LL cells, we conducted ELISA analysis and quantitative real-time PCR for VEGF. Low concentrations of VNR reduced VEGF secretion from 3LL cells in a concentration-dependent manner (Figure 2A). As shown in Figure 2B, treatment of 3LL cells with VNR resulted in significant decrease of VEGF mRNA. These results suggest that a low concentration of VNR inhibits not only VEGF secretion but also its mRNA expression in 3LL cells.

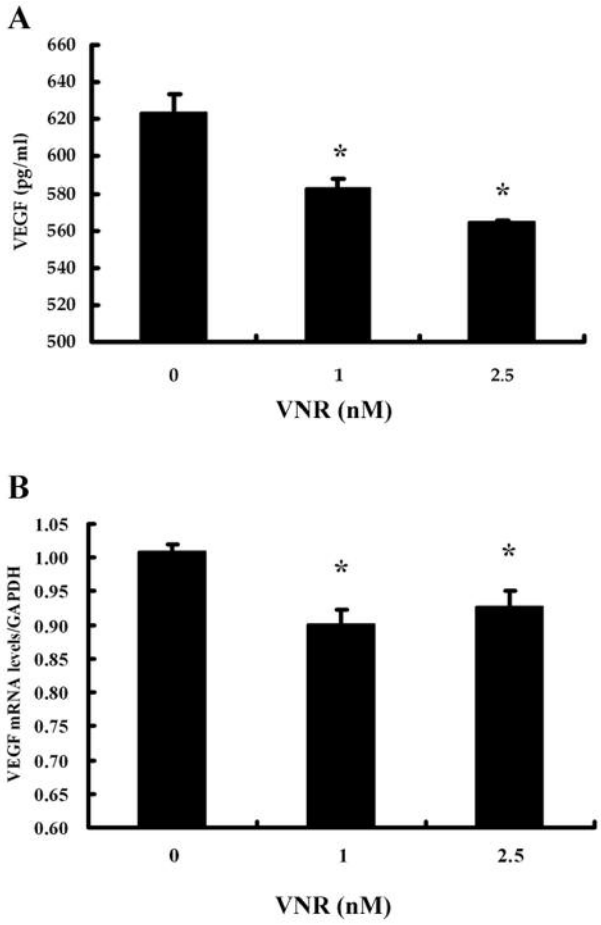

Figure 2. *A) Secretion of VEGF protein from VNR-treated 3LL cells. The cells were cultured with or without VNR for 48 h. Culture supernatants were collected from the control or VNR-treated 3LL cells, and then subjected to ELISA analysis. *p<0.05 versus 0 nM VNR. B) Expression of VEGF mRNA in VNR-treated 3LL cells. The cells were cultured with or without VNR for 24 h. VEGF mRNA expression levels in the control and VNR-treated 3LL cells were determined with quantitative real-time polymerase chain reaction using specific primer sets. The result is expressed as mean ± SD. *p<0.05 versus 0 nM VNR.*

Treatment with VNR reduces malignant pleural effusion. As shown in Figure 3A, administration of VNR completely inhibited formation of MPE in comparison with the saline-administered group. As expected, a significant reduction in intrapleural tumor weight was found in VNR-treated mice compared to that of the saline-administered mice. These findings indicate that VNR significantly inhibits MPE formation as well as tumor growth produced by 3LL cells.

Treatment with VNR inhibits tumor vascularization and VEGF expression. As shown in Figure 4A, the number of VEGF-positive cells was markedly reduced in the VNR-treated mice in comparison with that of the control mice. As

a next step, immunohistochemical staining for CD31 was performed to investigate tumor vascularization. As shown in Figure 4B, the number of CD31-positive vascular endothelial cells was significantly reduced in the VNR-treated mice compared to that of the control mice. These results suggest that VNR down-regulates VEGF expression resulting in suppression of tumorigenicity and MPE formation by the inhibition neovascularization.

VNR inhibits vascular permeability. In our study, VNR revealed a strong inhibitory effect for VEGF expression of 3LL cells *in vitro* and *in vivo*. Therefore, we investigated the effect of treatment with VNR on vascular permeability. As shown in Figure 5, rhVEGF165 induced significant vascular hyper-permeability compared with PBS in the control mice and this enhanced vascular permeability was significantly inhibited by treatment with VNR. These results imply that VNR can inhibit enhanced vascular permeability induced by VEGF.

Treatment with VNR prolongs survival. The median survival time (MST) of untreated control mice was 16.3 days. VNR treatment prolonged survival time, with an MST value of 23.7 days, in comparison with that of the control mice ($p<0.01$, log-lank test).

Discussion

In this study, we first demonstrated that treatment of MPE-bearing mice with VNR almost completely inhibited MPE production as well as intrapleural tumor growth. Immunohistochemical staining revealed that VNR markedly reduced neovascularization and VEGF expression of tumor tissues. We also revealed that VNR reduced *in vitro* lung cancer cell proliferation and VEGF expression.

VEGF is a multifunctional cytokine implicated in developmental, physiological, and pathological neovascularization (17, 18). VEGF also induces interendothelial gaps, endothelial fenestration and increases vascular permeability by activating cytoplasmic vesicular organelles in endothelial cells (19-21). Various kinds of tumor cells, including NSCLC cells, secrete VEGF (22). In patients with adenocarcinoma of the lung with MPE, greater than approximately tenfold VEGF levels were identified in the pleural fluid compared with that in matched sera (23). Yanagawa H *et al*. reported that VEGF levels were markedly increased in MPEs associated with lung cancer compared with those in pleural effusion caused by benign diseases, including heart failure and pulmonary tuberculosis (24). Inhibition of VEGF expression with antisense VEGF gene in the PC14PE16 human lung adenocarcinoma cell line, significantly reduced MPE formation in animal model (25). Likewise, inhibition of VEGF receptor phosphorylation reduced MPE formation in mice (1, 26). These results suggest that VEGF plays a critical role in the formation and pathogenesis of MPE and ascites in lung cancer (27, 28).

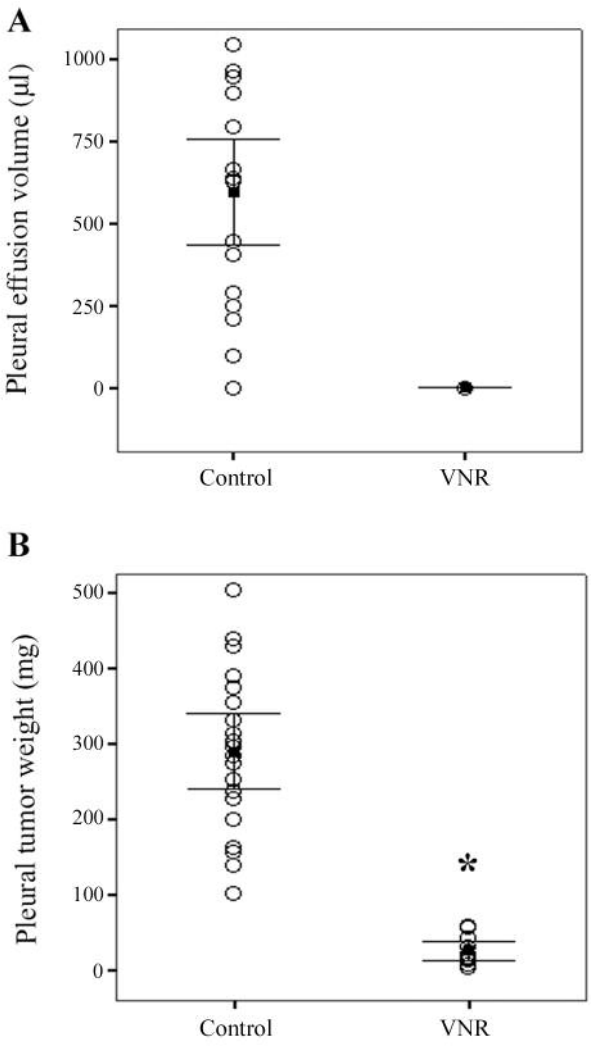

Figure 3. *Effect of VNR on the production of pleural effusions (A) and tumors (B). Ten thousand 3LL cells were directly injected into the pleural space of C57BL/6 mice. After 8 days, the mice were intravenously injected with saline (n=20) or VNR (30 mg/kg, n=12). The mice were sacrificed on day 13, and pleural tumor weight and pleural effusion volume were evaluated as described in the Material and Methods section. A, VNR completely inhibited production of malignant pleural effusion in mice. B, VNR significantly reduced pleural tumor weight, *p<0.0001 versus saline.*

In the present study, a high dose of VNR (5 and 10 nM) significantly inhibited 3LL cell proliferation *in vitro*. On the other hand, a low dose of VNR (1 and 2.5 nM) markedly reduced VEGF mRNA and protein expression in 3LL cells although it did not affect 3LL cell proliferation. *In vivo*, VNR revealed strong antitumor activity with a reduction of MPE production in our mouse model. Moreover, treatment of MPE-bearing mice with VNR significantly prolonged

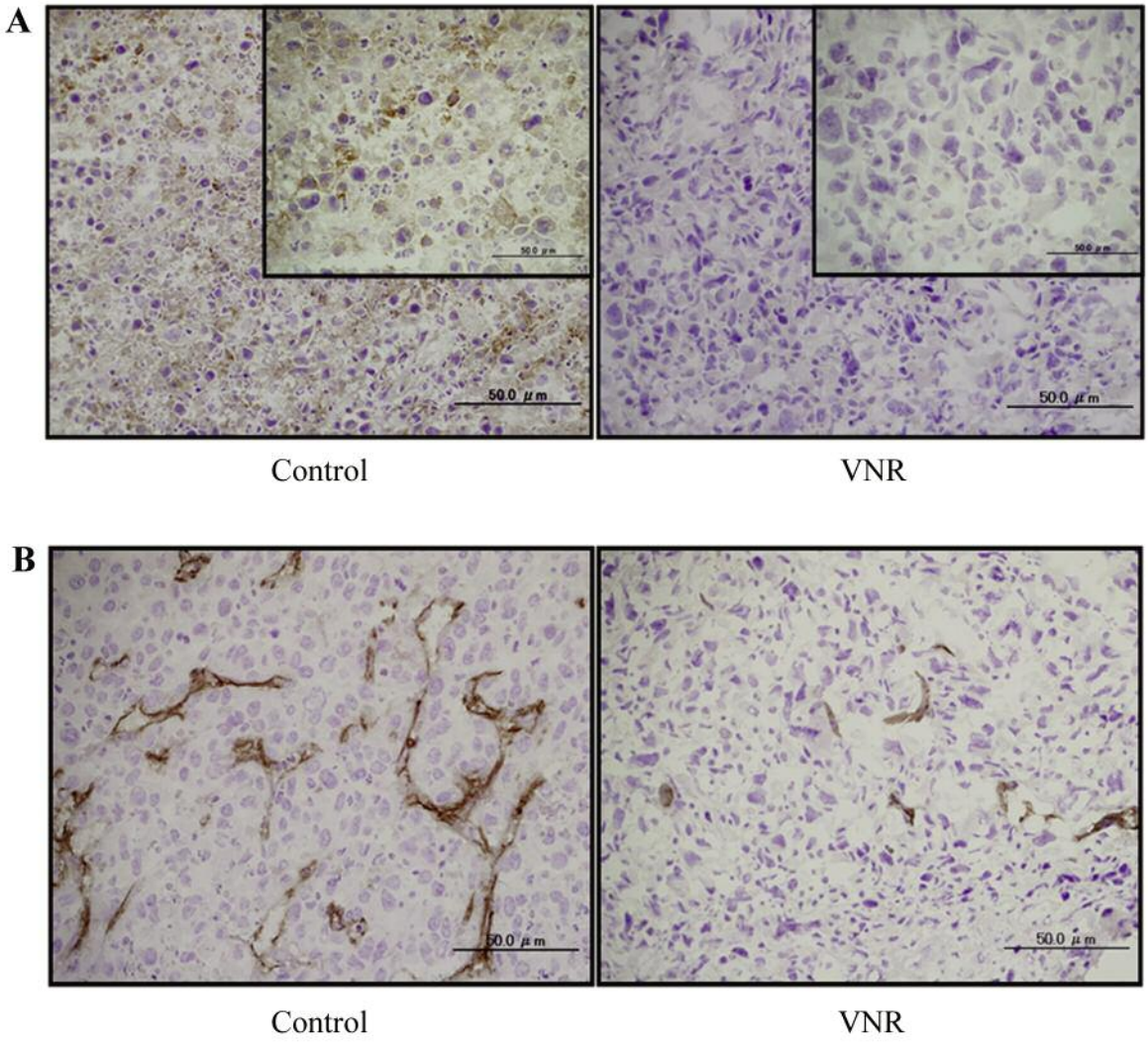

Figure 4. *A, Immunohistochemical staining for VEGF of tumor derived from the control and VNR-treated mice. Cryostat sections of tumors obtained from C57BL/6 mice were stained with anti-VEGF antibody (original magnification ×400). Insets are magnified views (×1000). B, Vascularization of tumors derived from the control and VNR-treated mice. Cryostat sections of tumors obtained from C57BL/6 mice were stained with anti-CD31 monoclonal antibody. Representative sections are depicted (×400).*

survival in comparison with that of the control mice. We also demonstrated that VNR markedly reduced neovascularization and VEGF expression on intrapleural tumor tissues with immunohistochemical staining. Furthermore, the Miles assay revealed that VNR significantly inhibits enhanced vascular permeability induced by VEGF. These results suggest that the mechanism of inhibitory effect of VNR on MPE production may be attributed not only to its antitumor activity but also to the down-regulation of VEGF expression.

To the best of our knowledge, what anticancer agents affect VEGF expression on cancer cells is still controversial. For example, cisplatin and doxorubicin repress VEGF mRNA expression in human ovarian cancer cells, while doxetaxel and paclitaxel do not (29). In contrast, Avramis *et al*. reported that doxetaxel inhibited VEGF secretion from human leukemia cells (30). With regard to VNR, there have been no studies describing its inhibitory effect on VEGF expression. This report appears to be the first which revealed that VNR suppresses VEGF expression and production in lung cancer.

Since VEGF is closely implicated in tumor blood vessel formation and permeability, targeting of VEGF in combination with cytotoxic agents has been considered as an effective therapeutic modality for cancer therapy, including of NSCLC. In fact, the antiangiogenic agent bevacizumab, a monoclonal antibody directed against VEGF, plus carboplatin and paclitaxel have been revealed to improve

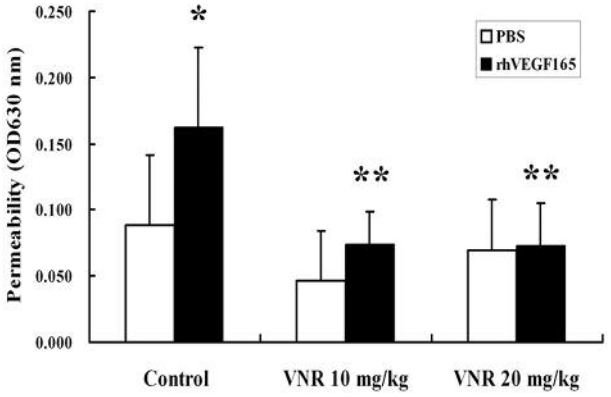

Figure 5. *Effect of treatment with VNR on vascular permeability. VNR (10 or 20 mg/kg) or phosphate buffered saline (PBS) were intravenously administered into nude mice. After 2 h, 50 μl of PBS or rhVEGF165 (30 ng/ml) were injected intradermally into nude mice that were preinjected with 0.5% Evans blue dye (200 μl). Thirty minutes later, the mice were sacrificed, and the skin was excised to extract the Evans blue dye. The absorbance of the extracts was determined at 630 nm with a spectrophotometer. The data represent the mean of sample from six mice; bars, SD. *p <0.05 versus Control PBS; **p<0.05 versus Control rhVEGF165.*

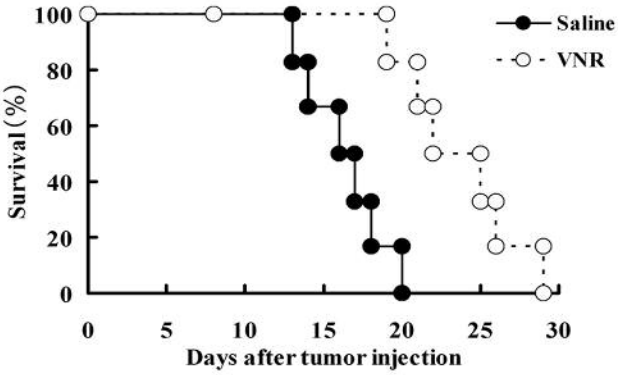

Figure 6. *Effect of VNR on survival of MPE-bearing mice. Ten thousand 3LL cells were directly injected into the pleural cavity of C57BL/6 mice. Eight days after injection, VNR (30 mg/kg) or saline was administered once intravenously. VNR significantly prolonged survival with the percentage of increase in life span (% ILS) of 44.9% .*

overall survival in patients with advanced nonsquamous NSCLC compared to carboplatin and paclitaxel alone (31). These results suggest that a chemotherapeutic regimen including VNR could be an ideal treatment modality for patients with lung cancer because it has been proven that VNR possesses both antiangiogenic and cytotoxic effects.

Patients with poor performance status often cannot be administered the full dose of anticancer agents because of their adverse toxic effects. However, among several anticancer agents, VNR monotherapy has been demonstrated to prolong survival and to improve quality of life of NSCLC patients with poor performance status (14). In addition, much lower doses of VNR, which do not show tumor reduction, might be sufficient to reduce or control MPE associated with lung cancer based on our results of this study. This issue needs to be addressed in the future.

In conclusion, we revealed that VNR administration suppressed MPE production in the mouse MPE model. VNR could be an effective chemotherapeutic agent for patients with MPE resulting from lung cancer. Since VNR was demonstrated to reduce tumor growth and down-regulate VEGF expression in our mice model, further studies are necessary to prove the superiority of VNR for patients with MPE associated with lung cancer.

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