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1192 Vol. 10, 1192–1200, February 1, 2004 Clinical Cancer Research

A Definitive Role of RhoC in Metastasis of Orthotopic Lung Cancer in Mice

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ABSTRACT

Purpose: **Lung cancer is a major cause of cancer death, and its incidence is increasing in the world. Conventional therapies remain less effective for metastases of lung cancer, leading to poor prognosis of this disorder. The present study investigates pathological roles of RhoC in metastasis of lung cancer using a clinically relevant mouse model of lung cancer.**

Experimental Design: **RhoA, RhoC, dominant-negative Rho (dnRho) or green fluorescent protein gene was retrovirally transduced to murine lung cancer cells. For *in vivo* study, these transduced cells were intrapulmonary inoculated in syngeneic mice, and subsequently, growth and metastasis were analyzed. Migration and invasion activities were further investigated by *in vitro* chemotaxic chamber assays. Expression levels and activities of certain matrix metalloproteinases (MMPs) were explored by reverse transcription-PCR and gelatin zymography.**

Results: **Metastasis of lung cancer in the animal model, as well as *in vitro* migration and invasion, were significantly enhanced or inhibited by overexpression of RhoC or dnRho, respectively, without affecting the growth of primary tumors. Expression levels of certain MMPs and the activity of MMP-2 were significantly enhanced or suppressed by overexpression of RhoC or dnRho, respectively.**

Conclusion: **RhoC plays a crucial role in metastasis of lung cancer. RhoC does not affect tumor growth but enhances the metastatic nature of lung cancer by not only stimulating cell motility but also up-regulating certain MMPs. Attenuation of RhoC activity may be a potential target in the development of a novel strategy for treating metastasis of lung cancer.**

INTRODUCTION

Lung cancer is the leading cause of cancer death and accounted for 28% of all cancer death in the United States (1–3). The 5-year survival rate for all lung cancer patients remains only 10–15% in the United States, and the incidence of lung cancer continues to increase in the world (1, 3). About 80% of lung cancers are non-small cell lung carcinoma (NSCLC). The current therapies, including chemotherapy and radiotherapy, are less effective for NSCLC; the response rates in NSCLC remain <50%, and complete regression is rare (4). On the other hand, small cell lung carcinoma has a more highly metastatic nature than NSCLC, leading to a 5-year survival rate of <8% (5). Thus, the poor prognosis of lung cancer, including small cell lung carcinoma and NSCLC, indicates an urgent need for the development of innovative therapies for treating lung cancer (6, 7).

In general, prognoses of cancer patients, including cases of NSCLC and small cell lung carcinoma, deeply correlate with their clinical stages, which reflect the degree of metastasis to regional lymph nodes and distant organs, as well as the expansion of the primary tumor (1), *e.g.*, lung cancer patients diagnosed with stage IV disease have a 2-year survival rate of <20% (1, 3). Thus, an inhibition of metastasis is a potential useful target in the development of a novel therapeutic strategy for treating cancer. From the biological viewpoint, tumor invasion and metastasis represent a complex process, in which numbers of molecules may be involved, and some crucial factors and mechanisms remain to be solved (8). Authors of a recent study using DNA arrays implied that several genes involved in extracellular matrix assembly or actin-based cytoskeletal reorganization might play an important role in the metastasis of melanoma cells (9). Furthermore, this study showed that RhoC might modulate the metastatic nature of melanoma (9). These findings encourage us to investigate the pathological role of RhoC in metastasis of lung cancer.

RhoC GTPase is a member of the Ras-homology family of small GTP-binding proteins and responsible for actin cytoskeletal reorganization during cellular motility (10, 11). Relatively small numbers of studies on RhoC and cancer have been reported to date, although molecular mechanisms of RhoC-dependent cytoskeletal organization have been extensively investigated from the biological viewpoint (10, 12). The involvement of RhoC has been implicated in only limited types of cancers, such as melanoma (9), inflammatory breast cancer

Received 9/24/03; revised 11/3/03; accepted 11/7/03.
Grant support: Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan.
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(13, 14), ovarian cancer (15), and ductal adenocarcinoma of the pancreas (16), but no study has yet been reported regarding RhoC and lung cancer. More importantly, all previous reports describing RhoC and cancer, including those just cited, focus solely on cell motility as the mechanism in RhoC-dependent enhancement of cancer metastasis (9). In the present study, we investigated the pathological role of RhoC using the clinically relevant mouse model of lung cancer, and a novel mechanism of RhoC in cancer metastasis, which is that RhoC apparently up-regulates matrix metalloproteinases (MMPs).

MATERIALS AND METHODS

Cell Lines. The murine lung cancer cell line Lewis lung cancer (LLC) was purchased from American Type Culture Collection (Rockville, MD) and cultured in 5% CO_2 at 37°C in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

Reverse Transcription-PCR. Total RNA was extracted from LLC cells using Sepazol RNA I super kit (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's protocol. For reverse transcription-PCR analysis, 1 μg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (Invitrogen Corp., Carlsbad, CA), and then 1/100 of the cDNA was subjected to PCR amplification by 38 cycles of 94°C for 30 s, each annealing temperature for 1 min and 72°C for 1 min using the primer sets described previously (16–18). Annealing temperatures were 58°C for *RhoA* and *RhoC* genes; 55°C for *MMP-2, MMP-9,* and *membrane-type (MT) 1-MMP* genes; and 45°C for the *tissue inhibitors of metalloproteinase (TIMP)-2* gene. The *hypoxanthine-guanine phosphoribosyltransferase* gene was amplified as an internal control with the same cycle and temperature as was used for each target gene using the following primer set: (*a*) sense 5′-CCTGCTGGATTACATTAAAGCACTG-3′; and (*b*) antisense 5′-AAGGGCATATCCAACAACAA-3′. The amplified cDNA was electrophoresed on 2% agarose gel containing ethidium bromide. The reproducibility of the results was confirmed by three independent experiments.

Retroviral Vectors and Gene Transduction. The retroviral vector plasmids pMIG-RhoA, pMIG-RhoC, pMIG-N19RhoA (a kind gift from Dr. R. Hynes), and pMX-green fluorescent protein (GFP; a kind gift from Dr. T. Kitamura) were transfected to the 293T cell-derived ecotropic retroviral packaging cell line Phoenix (a kind gift from Dr. G. Nolan) to produce retroviral vectors that express RhoA, RhoC, dnRho and enhanced GFP, *i.e.*, RV-RhoA, RV-RhoC, RV-dnRho, and RV-GFP, respectively (9, 19, 20). Supernatant containing each retroviral vector was collected at 48, 60, and 72 h after transfection and was used for three rounds of retroviral infection with LLC cells (21). Retroviral gene transduction efficiencies and transgene expressions were confirmed by flow cytometric analysis (FACStar; Becton Dickinson, Bedford, MA) of RV-GFP-infected cells and reverse transcription-PCR analysis of each transgene.

Animal Studies. Female C57BL/6 mice at 6–8 weeks old were purchased from Japan Slc, Inc. (Hamamatsu, Japan) and housed in cages in a temperature-controlled room on a 12-h light-dark cycle with free access to food and water. All animal studies were performed in accordance with the NIH guidelines as dictated by the Animal Care Facility at the Gifu University School of Medicine.

Mice received a percutaneous and intrapulmonary inoculation of the transfected LLC cells as described previously with some modification (6, 22). Briefly, after a small incision had been made in the skin on the left chest wall, a 29-gauge needle attached to a 0.5-ml syringe was directly inserted into the left lung at a depth of 3 mm, whereas the animal was under ether anesthesia. Subsequently, 1×10^3 retrovirally infected LLC cells mixed with Matrigel basement membrane matrix (Becton Dickinson) were injected into the left lung. The animals were sacrificed at 10, 14, and 21 days after tumor implantation; the sizes of primary tumors in the lung were measured, and the mediastinal lymph nodes were weighed. The volume of the primary tumor was calculated using the formula ½ × (long diameter) × (short diameter)^2.

Histopathology and Immunohistochemistry. Tissues were fixed in 10% formalin and embedded in paraffin, and 4-μm sections were stained with H&E to evaluate the morphology. For immunohistochemistry, 6-μm frozen sections were fixed in 4% paraformaldehyde and stained with rabbit anti-GFP antibody (Molecular Probes, Inc., Eugene, OR), Alexa Fluor 488-conjugated antirabbit IgG (Molecular Probes, Inc.), and Hoechst 33342 (Molecular Probes, Inc.).

***In Vitro* Cell Migration and Invasion Assays.** Cell migration and invasion assays were performed using 8-μm pore size Transwell Biocoat Control inserts and Biocoat Matrigel invasion chambers (Becton Dickinson), respectively, according to the manufacturer's protocol. In brief, 5×10^4 retrovirally transduced LLC cells were seeded on a transwell containing numbers of 8-μm pores covered with or without a thin layer of Matrigel basement membrane matrix for invasion or migration assay, respectively, which was set on a 24-well plate. Cells on the top surface of the transwell were removed by scrubbing 24 h after incubation, and the remaining cells on the bottom surfaces of the membrane were fixed and stained using Diff-Quick staining kit (International Reagents Corp., Kobe, Japan). Such invaded and migrated cells were counted and compared among groups. Individual experiments were done in duplicate and repeated four times.

Gelatin Zymography. Gelatin zymography was performed using a Gelatinzymo electrophoresis kit (Yagai Research Center, Yamagata, Japan) according to the manufacturer's protocol. In brief, 2.5×10^5 cells were seeded onto 10-cm dishes and incubated for 24 h in the serum-containing media. The cells were then rinsed twice and incubated in serum-free media for an additional 24 h. After the collected supernatants were centrifuged to remove contaminated cells and debris, they were mixed with sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel containing 2.5 mg/ml gelatin. The gel was washed and incubated in reaction buffer for an additional 36 h at room temperature. It was then stained with Coomassie Blue and subsequently immersed with destaining buffer for 3 h.

Statistic Analysis. Statistical significances between a control group (parental LLC cells) and each of the treatment groups (retrovirally transduced LLC cells) were determined using the unpaired Student t test. $P < 0.05$ was considered to have statistical significance.

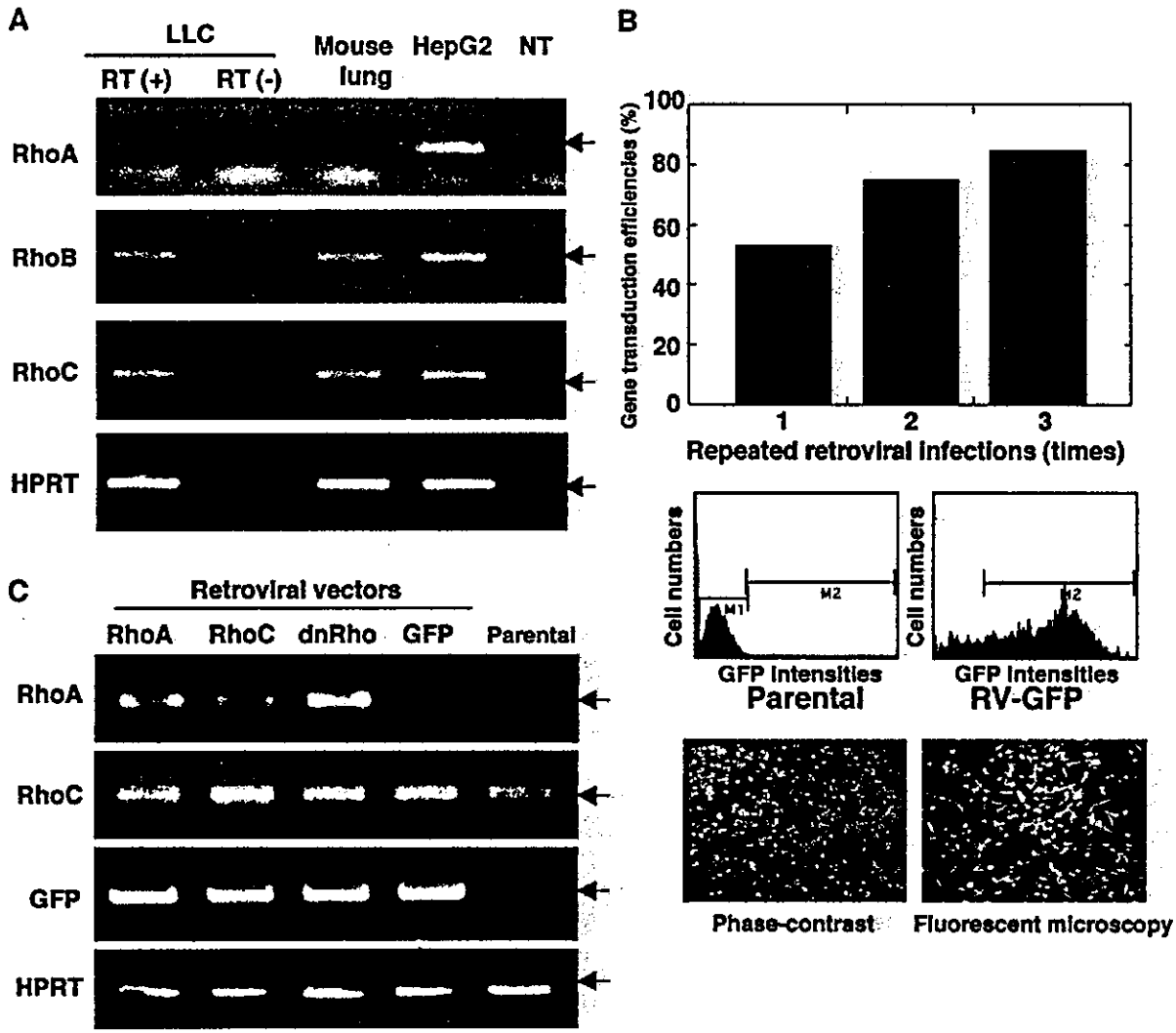

Fig. 1 Retroviral gene transduction and expressions of Rho family genes. *A*, endogenous mRNA levels in Lewis lung cancer (*LLC*) cells by reverse transcription (*RT*)-PCR analysis. RT(+) and RT(−) indicate PCR analysis with or without reverse transcription, respectively. *NT*, no template as a negative control. Total RNA extracted from HepG2 cells or mouse lung tissue was used for a positive control of PCR assay. *Hypoxanthine-guanine phosphoribosyltransferase* gene was amplified as an internal control gene. *B*, flow cytometric (in *top* and *center* figures) and fluorescent microscopic (in the *bottom* figures) analyses after retroviral infections in LLC cells. The percentages of green fluorescent protein (*GFP*)-positive cells after one, two, or three rounds of infection with RV-GFP are shown in the *top* figure. The *center* figures show the intensities and numbers of GFP-positive cells that received three rounds of infection with RV-GFP and those of parental LLC cells without any retroviral infections. The LLC cells after three rounds of infection with RV-GFP were also observed under the phase contrast and fluorescent microscopy (the *bottom* figures). *C*, mRNA expression levels of transgenes after three rounds of retroviral infection in LLC cells.

RESULTS

Retroviral Gene Transduction and Expressions of Rho Family Genes. Reverse transcription-PCR analysis demonstrated that parental LLC cells endogenously expressed RhoA, RhoB, and RhoC at relatively low levels (Fig. 1*A*). A flow cytometric analysis demonstrated that a single infection with RV-GFP resulted in 56% gene transduction efficiency in LLC cells (Fig. 1*B*). Two or three rounds of retroviral infection further increased gene transduction efficiencies in LLC cells ≤74 or 86%, respectively. Importantly, expression levels of transgene were various among individual cells after three rounds of retroviral infection; this situation is similar to *in vivo* gene transduction but different from the use of artificial homogenous cloned cells. After three rounds of infection with each retroviral vector, expression of each target gene was significantly enhanced (Fig. 1*C*). Cells infected with RV-RhoA, RV-RhoC, and RV-dnRho expressed certain levels of GFP because pMIX plasmids comprise GFP cDNA downstream of the encephalomyocarditis virus internal ribosomal entry sequence (23). Increases in intensities of PCR bands corresponding to RhoA after RV-dnRho infections may be reasonable because the primer sets for RhoA also

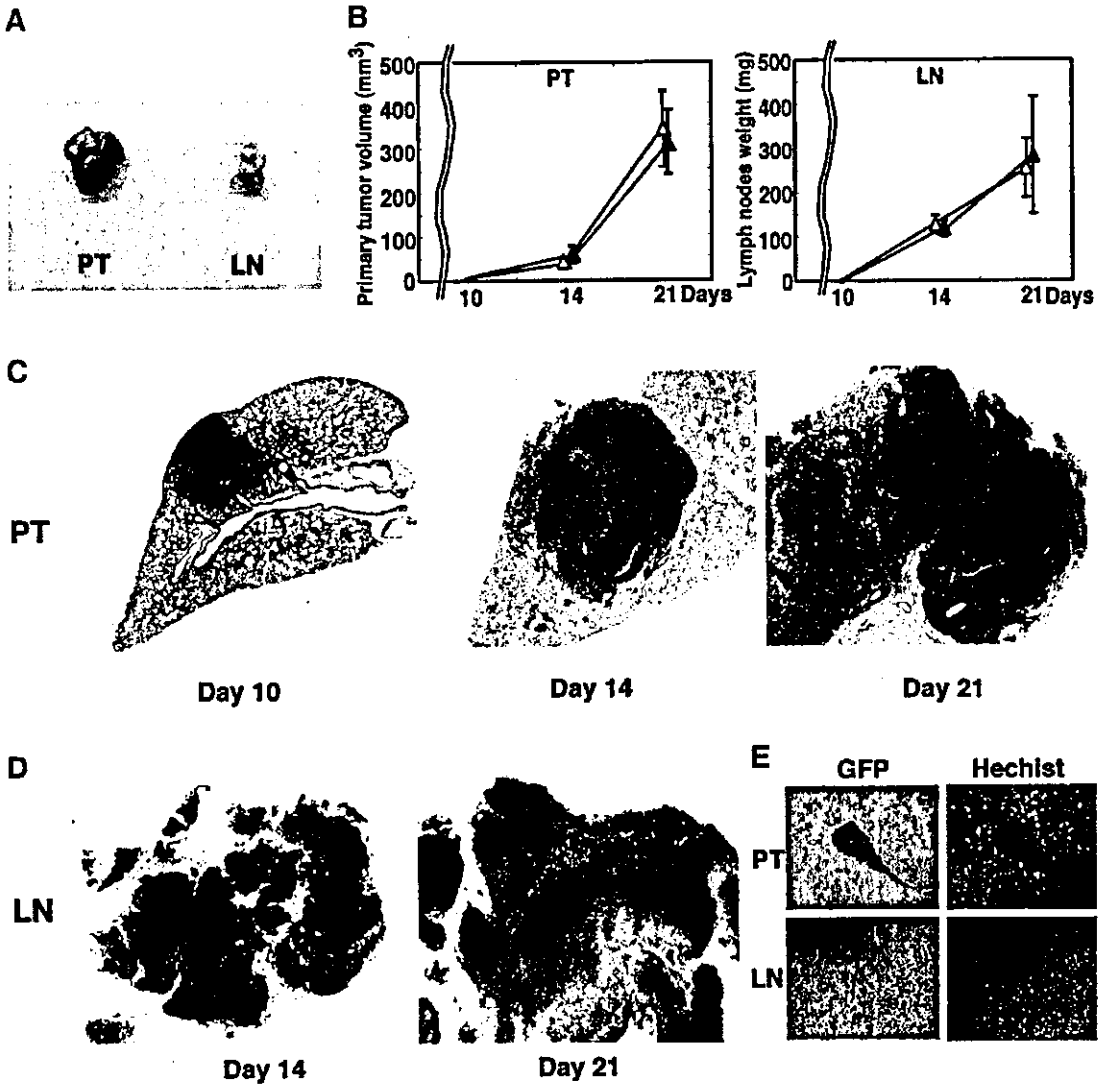

Fig. 2 The orthotopic lung cancer model in mice. A mouse received intrapulmonary inoculation of 1×10^3 of Lewis lung cancer cells into the left lung. *A*, the representative macroscopic picture of the primary tumor (*PT*) and mediastinal lymph nodes (*LN*) 21 days after the tumor implantation. In *B*, the primary tumor volume and lymph node weight were shown. △ or ▲, the groups receiving the parental Lewis lung cancer cells without any retroviral infection or those receiving the RV-green fluorescent protein (*GFP*)-infected Lewis lung cancer cells, respectively (five mice per group). *C* and *D*, the representative microscopic pictures of the PT (*C*) and LN (*D*) 10, 14, and 21 days after the tumor implantation. H&E staining. Original magnification: ×20. In *E*, frozen sections of the PT and LN 21 days after implantation of the RV-GFP-infected Lewis lung cancer cells were stained with anti-GFP and Hoechst 33342.

have affinity to the sequence of dnRho (only substitution from Thr to Asn at codon 19 of the *RhoA* gene; Ref. 19).

Establishment of an Orthotopic Lung Cancer Model in Combination with Retroviral Gene Transduction. The primary tumor nodule in the lung parenchyma was macroscopically recognizable ~10 days after intrapulmonary implantation of parental LLC cells, and it rapidly grew and expanded between days 14 and 21 (Fig. 2, *A* and *B*). On the other hand, metastases of tumors to mediastinal lymph nodes were not macroscopically recognizable on day 10; however, metastatic foci were conspicuous on day 14, and metastases were prominent on day 21 (Fig. 2, *A* and *C*). It should be noted that there were not any differences in the growth of primary tumors, the degree of metastases to the mediastinal lymph nodes, or histological findings between RV-GFP-infected and parental LLC cells (Fig. 2*A–D*). Moreover, GFP immunohistochemistry demonstrated stable transgene expression in not only the primary tumor nodule but also metastases in the mediastinal lymph nodes on day 21 (Fig. 2*E*). Thus, retroviral gene transduction in combination with the orthotopic lung cancer model has advantages in stable transgene expression and in the lack of artificial effects relating to retroviral gene trans-

duction, which enable us to estimate actual effects of transgenes under clinically relevant conditions.

RhoC Enhanced Metastases without Affecting Primary Tumor Growth *in Vivo*. We studied the effects of Rho family genes and the dominant-negative Rho (dnRho) gene for primary tumor growth and metastasis to the mediastinum lymph nodes using this new system (Figs. 3 and 4). Interestingly, there were no differences among groups in macroscopic sizes, volumes, or histological findings of the primary tumor during the 21 days of the experiment. On the other hand, metastases to mediastinal lymph nodes were drastically increased between 14 and 21 days after tumor implantation in the case of overexpression of RhoC. In contrast, overexpression of dnRho resulted in significantly smaller sizes and weights of the mediastinal lymph nodes on day 21 compared with parental and GFP-expressing LLC cells. Overexpression of RhoA or GFP did not change the weights of the lymph nodes. It should be noted that no differences were seen in histopathological findings of the metastatic tumor among groups, including groups of RhoC- and dnRho-expressing LLC cells (Fig. 3). Thus, RhoC was largely involved in the metastatic activity of lung cancer (LLC cells) without modulating the growth or morphological change of the primary tumor.

RhoC Enhanced Activities of Both Migration and Invasion of Lung Cancer *in Vitro*. To investigate the mechanism of RhoC-dependent metastasis, we carried out *in vitro* migration and invasion assays (Fig. 5). Overexpression of RhoC enhanced migration as well as invasion, although the effect for the latter was more prominent than that for the former. In contrast, overexpression of dnRho inhibited both migration and invasion, whereas overexpression of RhoA or GFP did not affect either. These findings indicate that RhoC may regulate the metastatic nature of lung cancer not only by stimulating cell motility (probably by modulation of the cytoskeletal structure that is well known) but also by directly enhancing the invasive activity, *i.e.*, the degradation of extracellular matrix.

RhoC Enhanced the Expression and Activity of MMPs. It is well known that MMP family proteins play central roles in cancer invasion relating to enzymatic degradation of extracellular matrix. Therefore, we examined mRNA expression levels and activities of the representative MMPs to assess whether the invasive effect of RhoC may result from modulation of MMP activities (Fig. 6). Overexpression of RhoC resulted in significant increases in mRNA expressions of MMP-2, MMP-9, MT1-MMP, and TIMP-2. In contrast, overexpression of dnRho decreased mRNA expression levels of MMP-2, MMP-9, MT1-MMP, and/or TIMP-2 to some degree (prominently, those of MTP-9 and MT1-MMP) in comparison with those of control RV-GFP-infected or parental cells. Zymographic analysis further and strongly confirmed this finding; not only pro (inactive) forms of MMP-2 and MMP-9 but also the active form of MMP-2 were significantly up-regulated by overexpression of RhoC. In contrast, dnRho decreased proMMP-2 and proMMP-9, although the inhibitory effect for active MMP-2 could not be assessed in this experiment because of the absence of detectable levels of active MMP-2 in control parental LLC cells. All these results suggest that RhoC may up-regulate MMPs at the transcriptional and/or enzymatic processional levels, leading to enhancement of the invasive activity of the LLC cells.

DISCUSSION

This is the first study to reveal clearly and experimentally that specifically RhoC, but not RhoA, plays a crucial role in the metastasis of lung cancer. Previous studies implied involvement of the Rho family, including Rho (*e.g.*, RhoA, B, and C), Rac, and Cdc42 proteins, in cancer (12), but the pathological role of each of the Rho family proteins was not clear, and the overall mechanisms in Rho-dependent oncogenic effects have not yet been fully clarified. Physiological functions of these family proteins have been largely investigated; they regulate a wide variety of cell function, especially cytoskeletal reorganization during cellular motility (10, 24). Importantly and interestingly, despite the high homology of the different Rho isoforms, RhoA, RhoB, and RhoC (25, 26), their physiological roles are distinct (24). Likewise, there is a possibility that a specific type of Rho isoform may be involved in the pathogenesis of lung cancer. In clinical studies, significant increases in RhoA in head and neck squamous cell (27), upper urinary tract (28), colon (29), breast (29), and lung (29) cancer and significant increases in RhoC in breast cancer (13, 14, 30) were shown, although other Rho isoforms were not investigated in these studies. Thus far, only four studies, to our knowledge, have compared the combined expression levels of RhoA, RhoB, and RhoC in cancer of human patients. Three of the studies demonstrated significantly higher levels of both RhoA and RhoC in ovarian carcinoma (15), bladder cancer (31), and breast tumors (32) than levels in nontumor tissues. It should be noted that only one of these four studies demonstrated a significant increase in RhoC only, and not in RhoA or RhoB, in ductal adenocarcinoma of the pancreas in human patients (16).

A majority of previous experimental studies on Rho and cancer used RhoA as the representative of the Rho family or the materials that might work on several Rho family proteins; based on such results, the pathological roles of the whole Rho family in cancer have been generalized and broadly speculated (19, 20). In addition, a variety of cancer cell lines has been used without any particular reason for their investigations, and based on the results, authors have roughly speculated about the function of the Rho family in a range of cancers. As a result, only one experimental study, to our knowledge, has investigated roles of the Rho family, specifically in lung cancer (33). However, the study used C3 exoenzyme that ADP-ribosylates Rho on Asn-41, *i.e.*, that might wok on both RhoA and RhoC, leading to indistinguishable assessments regarding predominant contribution of RhoA or RhoC (33). Thus, the pathological roles of RhoC in lung cancer have not yet been well defined from clinical or experimental studies; the present study for the first time presents convincing evidence that RhoC, but not RhoA, at least in the type of murine lung cancer cells we tested, modulates the metastatic nature of lung cancer.

Accumulating data concerning biological functions of the Rho family proteins have suggested that tumorigenesis, cell cycle impairment, inhibition of apoptosis, and stimulation of invasion and metastasis may be possibly the major pathological roles of the Rho family in cancer (12). Tumorigenesis was not our focus in the present study and may be explored elsewhere. It remains to be clarified whether RhoC may confer tumorigenesis on lung tissue, because early studies revealed involvements

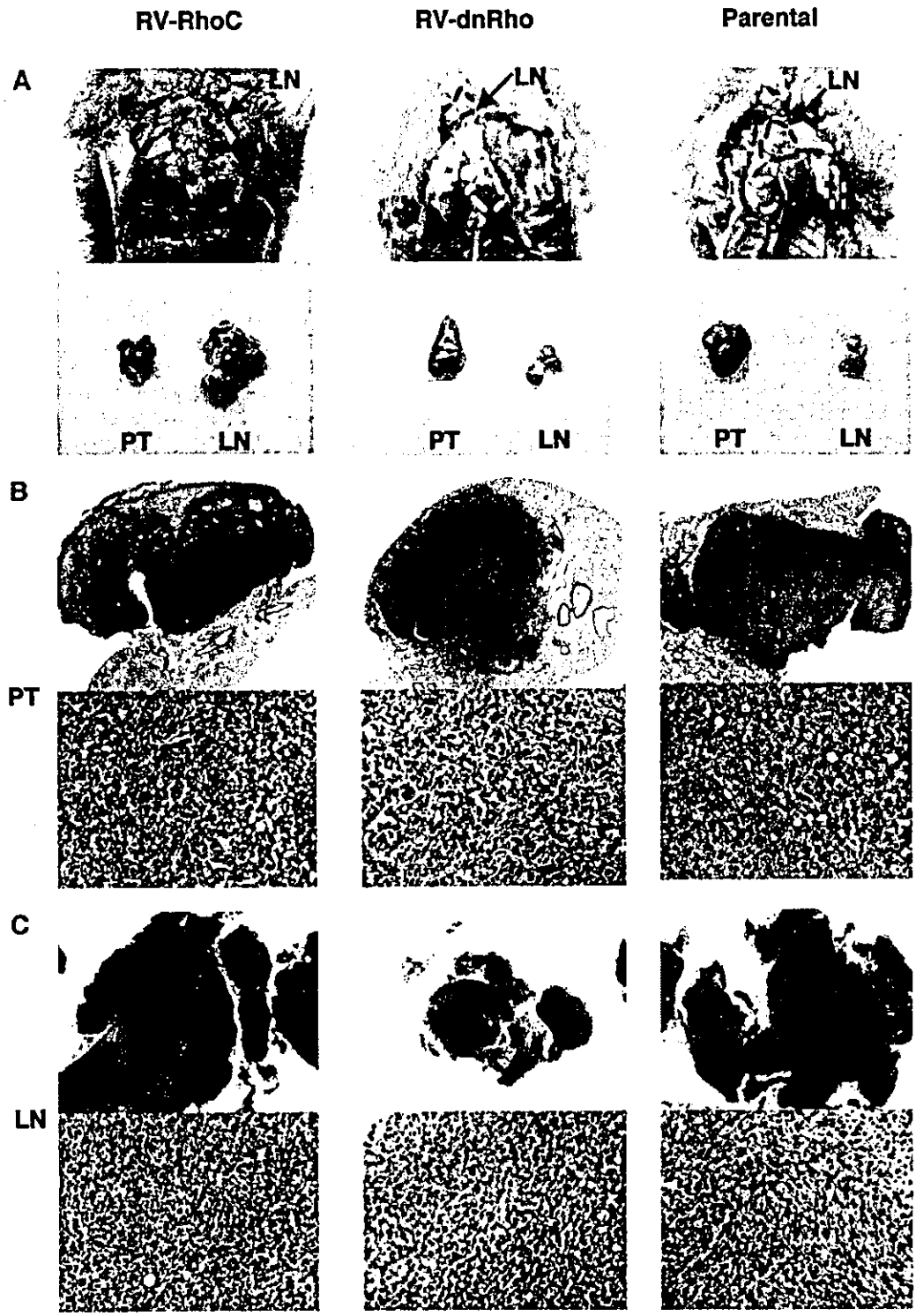

Fig. 3 The macroscopic and microscopic pictures of the primary and metastatic tumors in the mouse orthotopic lung cancer model. Lewis lung cancer cells (1×10^3 cells) that were beforehand infected with each retroviral vector were intrapulmonary inoculated into a mouse. In *A*, the representative macroscopic pictures of the primary tumor (*PT*) and metastatic tumor in the mediastinal lymph nodes (*LN*) 21 days after the implantation of RV-RhoC-infected, RV-dominant-negative Rho (*dnRho*)-infected, and parental Lewis lung cancer cells were shown. *B* and *C*, the microscopic pictures of the PT (*B*) and LN (*C*). H&E staining. Original magnification: ×20, the *top* pictures and ×200, the *bottom* pictures. The mice that received RV-RhoA- or RV-green fluorescent protein-infected Lewis lung cancer cells showed similar findings to the parental control (data not shown).

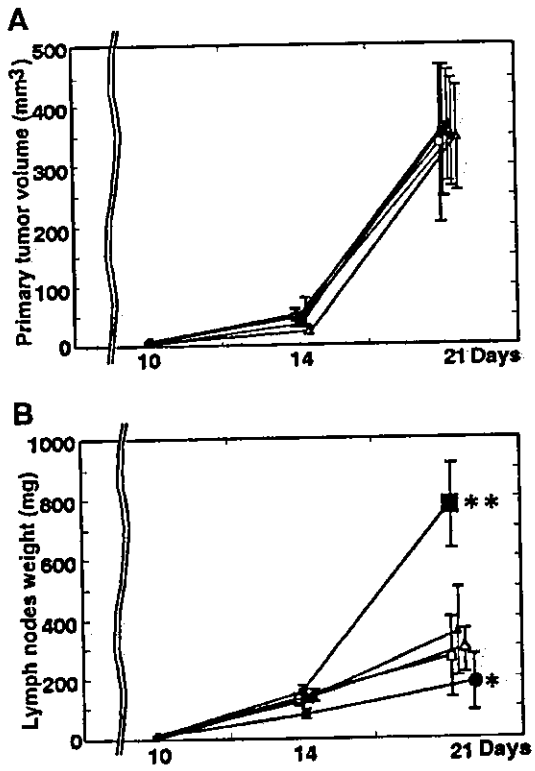

Fig. 4 The primary tumor volume (*A*) and lymph node weight (*B*). The protocol of the animal experiment was described in Fig. 3. □, ■, ●, △, and ▲, the RV-RhoA, RV-RhoC, RV-dominant-negative Rho, RV-green fluorescent protein, and parental (control) groups, respectively. Data represent means and SDs (n = 15–19/group). *, $P < 0.05$; **, $P < 0.01$ (Student's t test; each of treatment groups *versus* the parental control).

of only RhoA and other Rho family proteins in malignant transformation (19, 20, 34). On the other hand, involvements of Rho family proteins in cell cycle-related molecules and/or mitogenic signal transduction pathways have been extensively studied from biological viewpoints (10, 12). In addition, the relationship between Rho family proteins and apoptosis has recently been implicated (10, 12). Because disregulations of cell growth and cell death are the characteristic and essential nature of cancer, it has been speculated that increases in Rho proteins may be involved in the proliferation of cancer cells and/or their resistance to death signals. In the present study, however, neither the growth nor death of tumor cells in the primary tumor was affected by gene transduction of dnRho or RhoC. In contrast, overexpression of RhoC remarkably stimulated not only the metastasis of lung cancer in this clinically relevant animal model but also the activities of both migration and invasion in the *in vitro* assays. Moreover, gene transduction of dnRho inhibited the metastasis in the animal model and the activities of migration and invasion. Thus, it is likely that a number of molecules, various signal transduction pathways, and diverse biological effects relating to RhoC may mostly contribute to the activities of migration and invasion of cancer cells, *i.e.*, the metastasis in cancer diseases, but not to the enhancement of cancer cell growth or antiapoptotic effect in cancer cells.

It is reasonable to consider that RhoC is largely involved in migration, *i.e.*, cell motility, of tumor cells, because RhoC regulates actin cytoskeletal organization, which is believed to provide the driving force for cell migration (10). On the other hand, it has been largely unclear how Rho family proteins exhibit the invasive activity, and the exact mechanism of RhoC-dependent invasion is almost unknown. The present study for the first time explored the relationship between RhoC and MMPs in cancer and clarified the novel mechanism; RhoC apparently up-regulated both expression levels and activities of MMPs, leading to enhancement of the invasive activity. We examined MMP-2, MMP-9, and MT1-MMP, as well as TIMP-2, which were the representative MMPs and their antagonist that were largely involved in invasion by a variety of

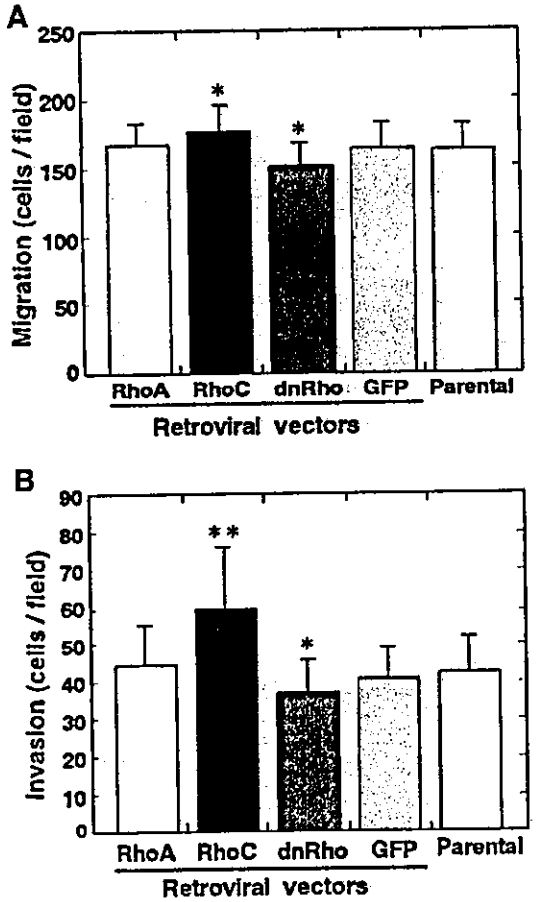

Fig. 5 *In vitro* migration (*A*) and invasion (*B*) activities. Lewis lung cancer cells received three rounds of infection with each retroviral vector as described in Fig. 1. The numbers of these cells on the bottom surface of transwell containing 8-μm pores covered with or without the Matrigel were counted as migration and invasion activities, respectively, 24 h after incubation. Data represent means and SDs of three independent experiments (n = 15/group in each experiment). *, $P < 0.05$; **, $P < 0.01$ (Student's t test; each of treatment groups *versus* the parental control).

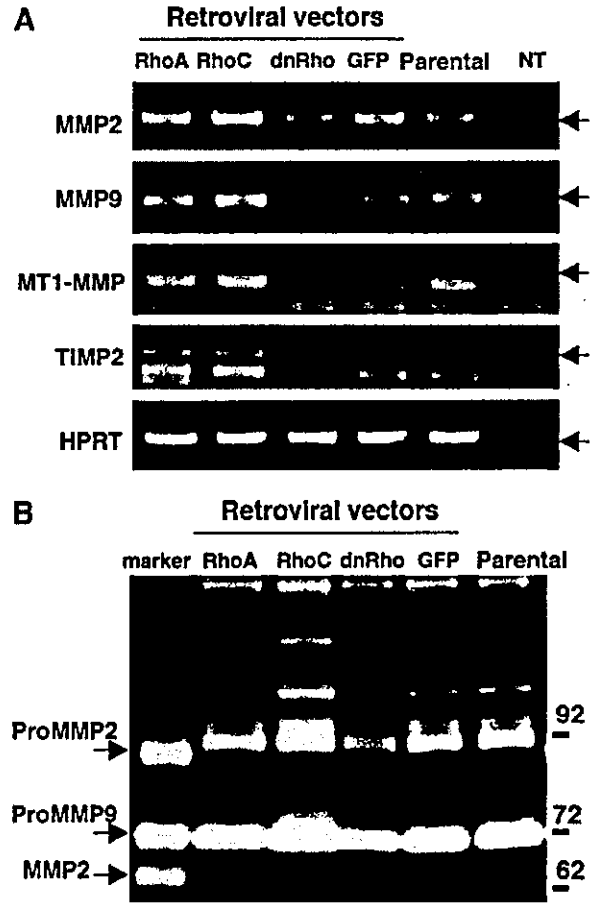

Fig. 6 Expression levels and activities of matrix metalloproteinases (*MMP*) after overexpressions of the Rho family proteins. Lewis lung cancer cells were infected with each retroviral vector as described in Fig. 1. *A*, reverse transcription-PCR analysis of certain MMPs. *NT*, no template. In *B*, the retrovirally gene-transduced Lewis lung cancer cells were incubated in serum-free media for 24 h, and the supernatants were electrophoresed on a 10% SDS-polyacryl-amid gel containing 2.5 mg/ml gelatin. The gel was incubated in reaction buffer for an additional 36 h and stained with Coomassie Blue.

cancers (35), including lung cancer in human patients (35–37). In fact, a clinical study demonstrated increases in MT1-MMP mRNA expressions and activation of MMP-2 in lung cancer, including small cell lung carcinoma and NSCLC, and a high correlation of the degree of MMP-2 activation with that of MT1-MMP mRNA expression and lymph node metastases (37). In the present study, gene transduction of RhoC significantly increased mRNA levels of MMP-2, MMP-9, MT1-MMP, and TIMP-2, and it activated MMP-2; gene transduction of dnRho apparently decreased mRNA levels of MMP-9 and MT1-MMP. Such RhoC-dependent transcriptional changes in MMP genes are new and interesting findings, and the detailed mechanisms, especially the transcriptional factors linking to RhoC, may be clarified in future studies. In this regard, the finding of an increase in mRNA levels of TIMP-2, an inhibitor for MT1-MMP and MMP-2, in this study should be noted. ProMMP-2 binds MT1-MMP using TIMP-2 as an adaptor by forming a trimolecular complex on the cell surface, and another MT1-MMP near the complex cleaves the propeptide bond of proMMP-2, leading to active MMP-2 (35). Although no study has investigated the mechanism of RhoC-dependent invasion, other recent studies have shown that RhoA and Rac1 could modulate the degradation and remodeling of the extracellular matrix by regulating the levels of either MMPs or their antagonists, TIMPs (38–40). In addition, Rac1 induced lamellipodia, and the MT1-MMP was localized there; Rac1 enhanced the activation of MMP-2 (35, 38, 41). The ruffled edge that forms the migration front is likely the site of the homo-oligomer formation of MT1-MMP that augments proMMP-2 activation (35, 41). Taken together, there is a possibility that RhoC may not only transcriptionally increase MMPs' expressions but also be responsible for localization of such MT1-MMP complex to migration edge. In this regard, a recent study showing a relatively stable association of MT1-MMP with actin cytoskeleton should be noted; CD44, which associated with actin within cells, localized at the migration front and was identified as a linker that mediated the association of MT1-MMP with actin (42).

Finally, it is encouraging that therapeutic effects of dnRho gene transduction were statistically significant in this clinically relevant lung cancer model. Somewhat milder phenotype after dnRho gene transduction than that of RhoC may possibly result from a relatively low expression level of endogenous RhoC in LLC cells and/or effective but incomplete inhibition of RhoC activity by the present dnRho gene construct. Nevertheless, the present results importantly suggest that attenuation of RhoC activity may be one of the potential targets in the development of novel strategies for treating metastasis of lung cancer.

In conclusion, RhoC apparently confers a metastatic nature on lung cancer without affecting the growth of primary tumors. One of the definitive mechanisms of the RhoC-dependent metastasis is up-regulating expressions and activities of MMPs.

ACKNOWLEDGMENTS

We thank Kazuko Goto and Hatsue Oshika in Gifu University for their technical assistance; Drs. Katsuyoshi Ichiki, Ikuo Saiki, and Takuro Misaki in Toyama Medical and Pharmaceutical University for their helpful comments on the animal model; and Drs. Richard Hynes in Massachusetts Institute of Technology, Toshio Kitamura in University of Tokyo, and Garry P. Nolan in Stanford University School of Medicine for providing materials.

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INTERNATIONAL JOURNAL OF ONCOLOGY 24: 549-558, 2004

Gene therapy eradicating distant disseminated micro-metastases by optimal cytokine expression in the primary lesion only: Novel concepts for successful cytokine gene therapy

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Received October 1, 2003; Accepted November 18, 2003

Abstract. The most serious problem in current gene therapy is that clinical applications have often led to unsatisfactory results. Here we show novel concepts and crucial factors that have been missing for successful cytokine gene therapy. A clinically-relevant mouse model of primary and micro-metastatic osteosarcoma was generated by subcutaneously and intravenously injecting murine osteosarcoma LM8 cells, in which adenoviral gene transduction efficiencies were extremely low; current therapies remain less effective for such disseminated micro-metastases. A single injection of adenoviral vector encoding interleukin-2 gene (Ad.IL-2) was given only into the established primary tumor. Notably, antitumoral immunity was successfully elicited by IL-2 secretion from connective tissues adjacent to the primary tumor, and this immunity not only suppressed primary tumor growth but also eradicated disseminated micro-metastases in distant organs. Most importantly, not only minimal side effects but also maximal therapeutic effects were exerted only in the case of injecting the optimal (i.e., not the highest) dose of Ad.IL-2, because spleen injuries caused by excessive levels of circulating IL-2 might diminish the therapeutic effect. Although the narrow range of the optimal therapeutic expression level of IL-2 may be crucial, it was feasibly determined by serum IL-2 levels. Thus, a crucial factor for successful cytokine gene therapy is not the high gene transduction efficiency in the tumor, which has been generally recommended, but the use of the optimal therapeutic expression level. In conclusion, just a single injection of Ad.IL-2 into a primary tumor lesion, which is feasible, not invasive and cost effective, is potently therapeutic for distant disseminated micro-metastases, as long as the optimal therapeutic level is monitored. These novel concepts, which contradict those of previous studies, warn researches about the possible problems with the ongoing clinical cytokine gene therapy.

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Key words: adenovirus, cytokine, gene therapy, micro-metastases, osteosarcoma

Introduction

Cytokine gene therapy, which elicits a systemic antitumoral immune reaction, is a potential candidate that may evade the present technical obstacle of the limited gene transduction in tumors in clinical situations and that may treat non-transduced and disseminated cancer cells. We have shown the therapeutic potential of cytokine gene therapy with interleukin-2 (IL-2), granulocyte macrophage-colony stimulating factor and interleukin-12 used alone or in combination with suicide gene therapy for a variety of cancers using their clinically-relevant animal models (1-5). IL-2, which is one of the most widely studied cytokines, stimulates cytotoxic and helper T lymphocytes (6,7), natural killer cells (8-10) and lymphokine-activated killer cells, leading to activation of non-specific and specific responses to non-immunogenic tumors (11,12). Our previous studies using animal models that possess clinical characteristics demonstrated that IL-2 gene therapy was therapeutic for peritoneal dissemination of hepatocellular carcinoma (2); however, neither therapeutic effects nor an antitumoral immune reaction were elicited by IL-2 gene therapy used alone (that is, not in combination with suicide gene therapy) for some solid tumors (1,4,5). In contrast, many previous studies by other investigators simply showed tumor regression as the sole therapeutic effect in

simple animal models that do not reflect clinical situations, and some clinical trials may have been potentially performed based on such insufficient preclinical analyses. Generally, the most serious problem in current cancer gene therapy is that clinical applications have led to unsatisfactory results, although promising data were shown in experiments (13-18). We have recently found some crucial factors for successful suicide gene therapy (19,20). Likewise, we hypothesize that some crucial factors for successful clinical application of cytokine gene therapy have been missing, and that the therapeutic potentials of cytokine gene therapy have not been maximally utilized for suitable disorders based on this therapy's advantages and characteristics.

Osteosarcoma is one of the most frequent bone cancers in children (21,22), and despite aggressive conventional treatments, more than 30% of patients develop metastases, most frequently in the lung, within the first year after an initial diagnosis (23). Only 14-30% of patients survive 5 years once they have had lung metastasis, while the survival rate is 60-80% in the case of a tumor being localized in the limb without metastasis (23-26). This poor prognosis indicates an urgent need for the development of innovative and novel therapies for lung metastasis of osteosarcoma.

In this study, we explored whether a single injection of the optimal dose of adenoviral vector (Ad) encoding IL-2 gene (Ad.IL-2) only into the primary tumor lesion was an effective treatment not only for the primary tumor but also for disseminated micro-metastases of osteosarcoma in distant organs, in clinical-relevant animal models. We chose this treatment not only because Ad-mediated IL-2 gene therapy for the treatment of osteosarcoma has not yet been studied, but also, more importantly, because this is a good model to assess our three hypotheses regarding cytokine gene therapy. IL-2 was chosen as a therapeutic gene in the present study in terms of being representative in cytokine gene therapy. Our first hypothesis was that the main target of disseminated micro-metastases in distant organs may be curable by treatment of only the accessible primary tumor. Our second hypothesis was that high gene transduction efficiency in tumors, the requirement of which has been believed to limit the clinical utility of cancer gene therapy approaches in general, may not be necessary for cytokine gene therapy. Our third hypothesis was that the optimal expression level of a cytokine would be one that could draw out the maximal therapeutic effect rather than produce the minimal adverse side effects, and that optimum level might not be the highest one. Along with preclinical findings for the treatment of lung metastasis of osteosarcoma, the novel concepts shown in the present study is crucial for ongoing and future cytokine gene therapy in general.

Materials and methods

Cell lines. LM8, a murine osteosarcoma cell line with high metastatic potential to the lung, which was generated from the Dunn osteosarcoma cell line (27), was given by Dr H. Yoshikawa (Osaka University) and Dr A. Uchida (Mie University). Human osteosarcoma cell lines, NOS-2, U2OS and SaOS, were obtained from the RIKEN Cell Bank (Tsukuba, Japan), American Type Culture Collection (Rockville, MD) and the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), respectively. All these cell lines were maintained in α-minimum essential medium (Sigma-Aldrich, Tokyo, Japan) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan).

Recombinant adenoviral vectors (Ads). Ad.IL-2, replication-deficient Ad encoding the murine IL-2 gene driven by a modified chicken ß-actin promoter with cytomegalovirus enhancer (CAG promoter), was given by Dr H. Hamada (Sapporo Medical College) through the RIKEN Gene Bank (Tsukuba, Japan). Ad.LacZ and Ad.dE1.3, which contain the LacZ gene and no gene in place of the murine IL-2 gene, respectively, were generated as previously described (1). All Ads were amplified in 293 cells, purified on CsCl gradients twice and desalted by desalting column (Econo-Pac® 10DG, Bio-Rad Laboratories, Hercules, CA). The titer of Ad (plaque-forming unit (pfu)/ml) was measured by a plaque assay on 293 cells.

Adenoviral gene transduction efficiency in vitro. To determine adenoviral gene transduction efficiency in LM8, NOS-2, U2OS and SaOS cells *in vitro*, $3x10^5$ of these cells were infected with Ad.LacZ at various multiplicity of infection (MOI) for 1 h and media were sequentially replaced with fresh media. The cells were incubated for 48 h at 37°C and sequentially fixed with 0.5% glutaraldehyde and stained with a solution containing 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-Gal, Wako Pure Chemical Industries Ltd., Tokyo, Japan), 13 mM $MgCl_2$, 15 mM NaCl, 44 mM HEPES (pH 7.4), 300 mM potassium ferricyanide and 300 mM potassium ferrocyanide. To evaluate adenoviral gene transduction efficiency, X-Gal-positive cells and total cells were counted in 20 microscopic fields under magnification x40, and the percentage of Ad-mediated gene transduction was calculated.

Animal model and experimental schedule. Our experimental schedule for working with animals is shown in Fig. 2. LM8 cells ($1x10^7$ cells) were injected subcutaneously into the back of 5-week-old male C3H/He mice. Subsequently, LM8 cells ($2x10^5$ cells) were injected via the tail vein at 6 days after tumor inoculation when the subcutaneous tumor became 5-6 mm in diameter, leading to 100% incidence of lung metastasis. At day 7 (1 day after intravenous injection of LM8 cells), all mice were randomly divided into 5 groups (5 mice in each group), and a single injection of Ad was given only into the subcutaneous primary tumor in 50 μl of 10 mM Tris-HCl (pH 7.4)/1 mM $MgCl_2$/10% (vol/vol) glycerol/Polybrene (20 μg/ml). Because all mice died several days after an injection of $3x10^8$ pfu of Ad.IL-2 into the primary tumor in a preliminary experiment, groups received the following respective injections in the present experiment: i) Ad.IL-2 ($1x10^8$ pfu), ii) Ad.IL-2 ($3x10^7$ pfu) + Ad/dE1.3 ($7x10^7$ pfu), iii) Ad.IL-2 ($1x10^7$ pfu) + Ad.dE1.3 ($9x10^7$ pfu), iv) Ad.dE1.3 ($1x10^8$ pfu) and v) vehicle. To ensure that the biological effect on tumor growth was due to the action of

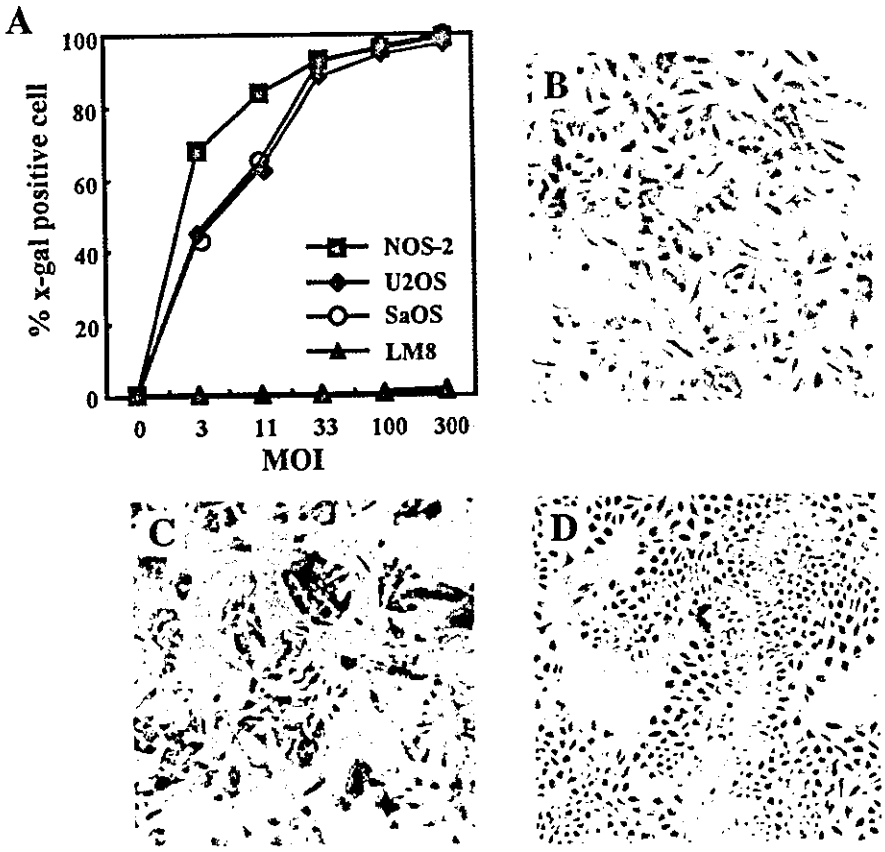

Figure 1. *In vitro* adenoviral gene transduction efficiencies in murine (LM8) and human (NOS-2, U2OS and SaOS) osteosarcoma cells. Tumor cells were infected with Ad.LacZ at various multiplicity of infection (MOI), followed by X-Gal staining. Adenoviral gene transduction efficiency in each tumor cell line at each MOI is shown (A). Representative pictures of SaOS (B), NOS-2 (C) and LM8 (D) cells at an MOI of 300 showed 100, 100 and 0.25% of adenoviral gene transduction efficiency, respectively. Original magnification x40.

the IL-2 gene and not the Ad doses, Ad.dE1.3, which contains no therapeutic gene, was used as a control and for adjusting the total Ad dose up to $1x10^8$ pfu in all four Ad groups. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Research Committee of Kurume University.

Adenoviral gene transduction efficiency in vivo. To evaluate the adenoviral gene transduction efficiency in tumors *in vivo* and the distribution of Ad around tumors and in other distant organs, Ad.LacZ ($1x10^8$ pfu) was injected into the subcutaneous tumor at 1 day after intravenous injection of LM8 cells. Three days later, mice were sacrificed, and the subcutaneous tumor, lung, liver, spleen and kidney were harvested to assess LacZ gene expression. Tumors and these organs were frozen in Tissue-Tek® OCT compound (Sakura, Torrance, CA), and sections were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde for 30 min. The sections were washed 3 times with PBS and assayed for ß-galactosidase activity by incubating the slides in X-Gal staining solution.

Assessment of therapeutic and adverse effect. To assess the therapeutic effect in primary tumors, we measured each tumor periodically and calculated its volume using the following formula: volume = long axis x (short axis)^2 x 0.5236 (28). Mice were sacrificed at 28 days after an Ad injection, and histologic examination was performed.

To analyze inhibitory effects on metastases in distant organs, the lung, liver, spleen and kidney were collected from each mouse at the time of sacrifice (at 28 days after Ad injection). First, metastases in these organs were carefully estimated by macroscopic observation. Subsequently, these tissues were fixed in 10% buffered-formalin, embedded in paraffin, cut into 4-µm samples and stained with hematoxylin and eosin, and then histopathological analysis was performed. For analyses of lung metastases, the number of nodules was microscopically counted in 200 fields under magnification x100.

To estimate adverse side effects, the lung, liver, spleen and kidney were collected from each mouse at the time of sacrifice (28 days after Ad injection). In another experiment, these organs were collected at 3 days after Ad injection on the same schedule as is shown in Fig. 2. The histopathological findings of these tissues were scrupulously observed and estimated under microscopy, and the degrees of injuries in these organs were scored.

IL-2 enzyme-linked immunosorbent assay (ELISA). Blood was collected from all mice that had received primary and metastatic osteosarcoma on the same schedule as is shown in Fig. 2, at 3 days after Ad injection into the primary tumor, and serum was obtained by centrifugation of blood at

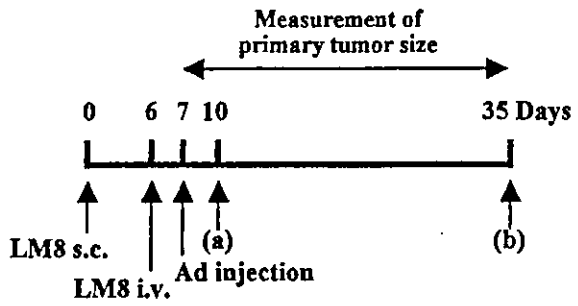

Figure 2. Schedule of animal experiments. LM8 cells were injected subcutaneously (s.c.) and intravenously (i.v.) in mice on days 0 and 6 to generate primary and metastatic tumors, respectively. Each Ad was injected only into the primary tumor on day 7. Mice were sacrificed on day 10 (a) for analyzing *in vivo* adenoviral gene transduction efficiency (Fig. 3), the histopathology of the primary tumor and distant organs (Fig. 6 and Table I) and serum IL-2 levels (Fig. 7). In another experiment, mice (n=5 in each group) that had received an inoculation of tumor cells and Ad injection on the same schedule were sacrificed on day 35 (b) for macroscopically and microscopically analyzing primary and metastatic tumors and other distant organs (Figs. 4 and 5), following macroscopic measurement of primary tumor size every 4 or 5 days (Fig. 4A).

3,000 rpm for 10 min. Serum mouse IL-2 levels were measured by ELISA according to the manufacturer's protocol (Cytoscreen®, BioSource International, Inc., Camarillo, CA). Briefly, serum and standards in which the IL-2 concentration had been predetermined were transferred and incubated in the wells, which were coated with an antibody specific for mouse IL-2, followed by the addition of a biotinylated second antibody. After incubation and the removal of excess second antibody, Streptavidin-peroxidase was added and the samples were incubated. After the samples were washed to remove unbound enzyme, a substrate reaction was added, leading to the production of color. We used a plate reader to measure the absorbance of serum samples and standards at 450 nm, and we calculated the IL-2 concentration from the plotted standard curve.

Results

Low adenoviral gene transduction efficiency in tumor in vitro and in vivo. In vitro adenoviral gene transduction efficiency in LM8 cells was extremely low, and it was only 0.25% at an MOI of 300, at which adenoviral gene transduction efficiency was 100% in all three human osteosarcoma cells, that is, NOS-2, U2OS and SaOS cells (Fig. 1).

An intratumoral injection resulted in predominant gene transduction in connective tissues adjacent to tumors while less than 1% of tumor cells in a tumor nodule received transgene (Fig. 3A). The potential leak of Ad from tumor cells did not result in significant distribution of gene transduction in other distant organs, that is, there was almost none in the kidney, lung and spleen and there were less than 1% of hepatocytes in the liver (Fig. 3B-D).

Growth inhibition of the primary tumor. We generated a good syngeneic and clinical-relevant animal model for not only correctly assessing the effects of immunomodulatory therapy but also exploring our hypotheses, using highly metastatic and non-immunogenic LM8 cells, in which adenoviral gene transduction efficiency was extremely low. The growth of primary tumors was suppressed to various degrees in all three groups of mice receiving different dosages of Ad.IL-2 (Fig. 4A). Interestingly, the best outcome was obtained only when the medium dose of Ad.IL-2

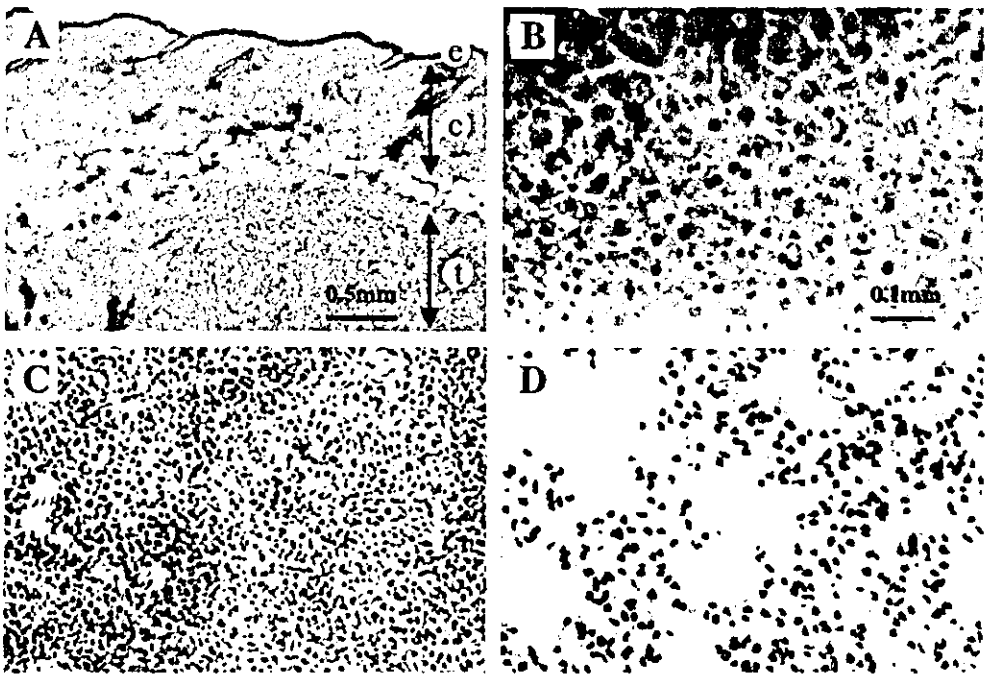

Figure 3. *In vivo* adenoviral gene transduction efficiencies in tumor and distant organs by Ad.LacZ injection into subcutaneous tumor nodules in mice. Representative pictures of an X-Gal-stained primary tumor (A) showing predominant gene transduction in a small portion of the tumor and connective tissues adjacent to the tumor nodule (e, epidermis; c, connective tissues in dermis and subcutaneous tissue; t, tumor). Less than 1% of hepatocytes were X-Gal positive (B), but no apparent X-Gal-positive cells were seen in the spleen (C) or lung (D). Original magnification x40 (A) and x200 (B-D).

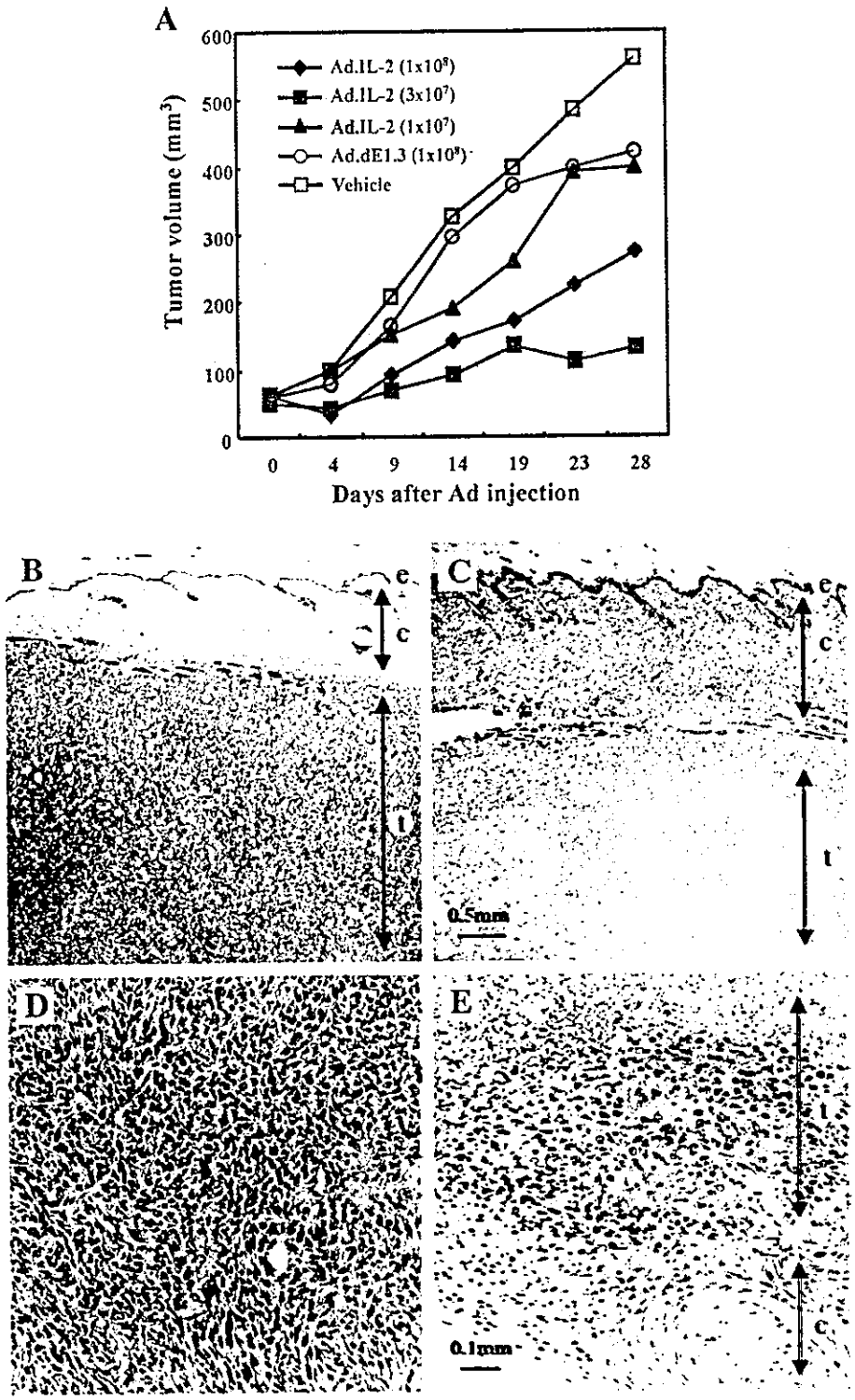

Figure 4. Therapeutic effect in primary tumors. Tumor growth was inhibited in mice that were treated with Ad.IL-2, and the best outcome was seen in the case of using the medium dose ($3x10^7$ pfu) (A). H&E-stained histology sections of primary tumor lesion in mice at 28 days after Ad injection (on day 35) showed prominent tumor death and remarkable infiltration of inflammatory cells in mice treated with $1x10^7$ pfu of Ad.IL-2 (C and E). In contrast, minimal tumor cell death and no apparent infiltration of inflammatory cells were seen in mice that were treated with Ad.dE1.3 (B and D) (e, epidermis; c, connective tissues in dermis and subcutaneous tissue; t, tumor). Original magnification was x40 (B and C) and x200 (D and E).

($3x10^7$ pfu) was administered. This inhibitory effect was significantly diminished when the 3-fold lower dose of Ad.IL-2 ($1x10^7$ pfu) was used. In addition, notably, a higher dose of Ad.IL-2 ($1x10^8$ pfu) did not result in further enhancement but rather diminished the inhibitory effect on primary tumors.

In contrast to absence of a necro-inflammatory reaction in any of the control mice (Fig. 4B and D), the primary tumor lesion in mice receiving Ad.IL-2 injection histologically exhibited prominent tumor cell death and marked infiltration of various kinds of inflammatory cells, including lymphocytes, granulocytes and macrophages, in the remaining tumor

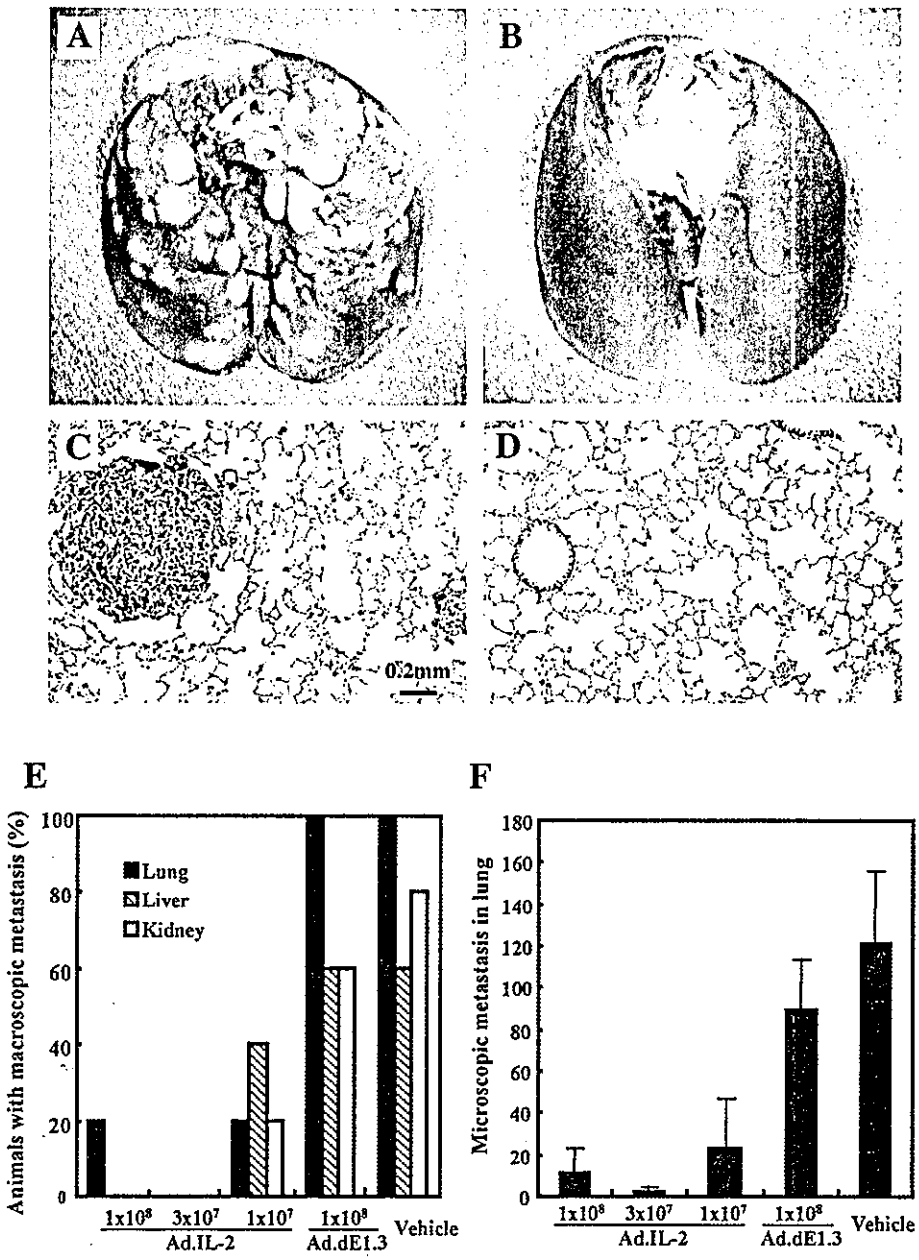

Figure 5. Therapeutic effects in distant organs. Macroscopic pictures (A and B) and H&E-stained microscopic sections (C and D) of lung tissue at 28 days after Ad injection into the primary tumor are shown. The lung was widely and diffusely invaded by many metastatic nodules consisting of osteosarcoma cells in control mice that were treated with Ad.dE1.3 (A and C). Such metastases were strongly inhibited in mice treated with $3x10^7$ pfu of Ad.IL-2 (B and D). Original magnification x100 (C and D). Morphometric analyses of metastases in distant organs (E and F). The percentages of mice, in which tumor nodules were macroscopically recognized in each distant organ, are shown (E). Numbers of tumor nodules in the lung was microscopically counted in 200 fields under magnification x100 (F). In both analyses, the best outcome was seen in mice that received an injection of $3x10^7$ pfu of Ad.IL-2 into the primary tumor.

cells or in connective tissues adjacent to the primary tumor (Fig. 4C and E).

A dramatic inhibition of metastases in other distant organs. In contrast to the high incidence of disseminated metastases in control mice (Fig. 5A and C), metastases in distant organs were significantly suppressed by an injection of Ad.IL-2 only into the primary tumor in treated mice (Fig. 5B, D and E). The best outcome was seen when the medium dose of Ad.IL-2 ($3x10^7$ pfu) was injected into the primary tumor, showing no macroscopically recognized metastasis in the lung, liver or kidney, as an inhibitory effect for metastases was completely consistent with that for the primary tumor in each Ad.IL-2-treated group.

Important and encouraging findings are that the inhibitory effect for lung metastasis was very strong in all groups of mice receiving Ad.IL-2, including the lowest dose of Ad.IL-2 ($1x10^7$ pfu), by which the inhibitory effect for the primary tumor was moderate. Both the number and the size of microscopically recognized tumor nodules in the lungs of mice receiving Ad.IL-2 were significantly smaller than those in control mice (Fig. 5F).

Table I. Injuries of major organs after intratumoral Ad injection.

Group	Liver		Spleen		Kidney		Lung	
	3 days	4 weeks	3 days	4 weeks	3 days	4 weeks	3 days	4 weeks
Ad.IL-2 (pfu)								
$3.0x10^8$	++~+++	ND	+++	ND	-	ND	+	ND
$1.0x10^8$	+	+	++	-	-	-	-	-
$3.0x10^7$	±	-	-	-	-	-	-	-
$1.0x10^7$	±	-	-	-	-	-	-	-
Ad.dE1.3	±	-	-	-	-	-	-	-
Vehicle	-	-	-	-	-	-	-	-

Degrees of injuries: +++, severe; ++, moderate; +, mild; ±, no apparent injury but mild inflammation; -, intact. ND, not determined because of death of mice.

Adverse side effects. Kidneys were not apparently damaged in any of the mice, including Ad.IL-2-treated mice. While lung damage was not significant at 3 days or at 28 days after $1x10^8$ pfu or less than $1x10^8$ pfu of Ad.IL-2 was administered into primary tumors, some of the mice treated with $3x10^8$ pfu of Ad.IL-2 showed a moderate degree of interstitial pneumonia at 3 days after Ad injection, consisting of infiltration of inflammatory cells in swollen alveoli (Table I).

In contrast, the liver and spleen apparently showed severe damage at 3 days after an injection of a high dose of Ad.IL-2 ($1x10^8$ pfu or $3x10^8$ pfu) into the primary tumor (Fig. 6 and Table I). Mice receiving the highest dose ($3x10^8$ pfu) of Ad.IL-2 showed that submassive hepatocyte death with marked infiltration of various kinds of inflammatory cells. The liver damage in mice receiving $1x10^8$ pfu of Ad.IL-2 was somewhat milder but still apparent, while mice receiving $3x10^7$ pfu or $1x10^7$ pfu of Ad.IL-2 or $1x10^8$ pfu of Ad.dE1.3 revealed a very mild necro-inflammatory reaction in the liver. Notably, all inflammatory reactions and damage to the liver were much milder in all mice at 28 days than those at 3 days, and even mice receiving an injection of $1x10^8$ and $3x10^7$ pfu of Ad.IL-2 revealed very mild and almost no apparent necro-inflammatory reaction in the liver, respectively, at 28 days after Ad injection.

The spleen was also severely damaged at 3 days after an injection of $3x10^8$ pfu or $1x10^8$ pfu of Ad.IL-2 into the primary tumor, histologically showing that the structure was deteriorated, and a necrotic area was widely seen in the white pulp. On the other hand, the spleen injury was not apparent when $3x10^7$ pfu or $1x10^7$ pfu of Ad.IL-2 was injected. Interestingly, these injured spleens were almost completely restored to normal tissue and no apparent necrotic area was seen at 28 days after an injection of any dosage, including $3x10^8$ pfu, of Ad.IL-2.

Thus, the liver and spleen were the main injured organs at an acute phase, however, such inflammatory reaction and injuries were remarkably diminished and tissue was restored relatively quickly once mice survived.

Serum IL-2 levels. Increases in serum IL-2 levels were well parallel to increases in the administered dose of Ad.IL-2 into the primary tumor. The serum IL-2 levels were 1.9±2.0 and 0.3±0.4 ng/ml when $1x10^8$ and $3x10^7$ pfu of Ad.IL-2 were injected into the primary tumor, respectively (Fig. 7). Thus, around 0.3 ng/ml and at least 1.9 ng/ml of serum IL-2 levels may be the indicative levels for achieving the best clinical outcome and for inducing severe side effects, respectively, in this mouse model.

Discussion

This is the first report that not only describes Ad.IL-2 gene therapy for osteosarcoma but also reveals novel and crucial concepts for successful cytokine gene therapy.

The first concept that we revealed is that disseminated micro-metastases in distant organs may be feasibly treatable by an injection of Ad.IL-2 into the primary tumor without any troublesome treatment directly in metastatic tumors. The present approach to the treatment of a primary lesion of osteosarcoma is potentially useful in terms of low invasiveness and improved quality of life as well as being therapeutically effective. The loss of limbs or joints resulting from traumatic surgical resection of a primary tumor largely degrades the quality of life in osteosarcoma patients, especially in children (29,30). Furthermore, the potential therapeutic effects for the treatment of metastatic tumors in distant organs, emerging from treatment of the primary tumor, are rather attractive in terms of treatment for life-threatening disorders. Patients whose primary tumor is controlled by conventional treatment may subsequently encounter and suffer from disseminated lung metastases, for which current therapy is less effective, and to which efficient gene transduction is not feasible using current vector systems. In contrast, even some of the tumor cells remaining at the primary site after IL-2 gene therapy may be treatable or controllable by current therapies if the inhibition of lung metastasis can be ensured. Thus, while the therapeutic effect

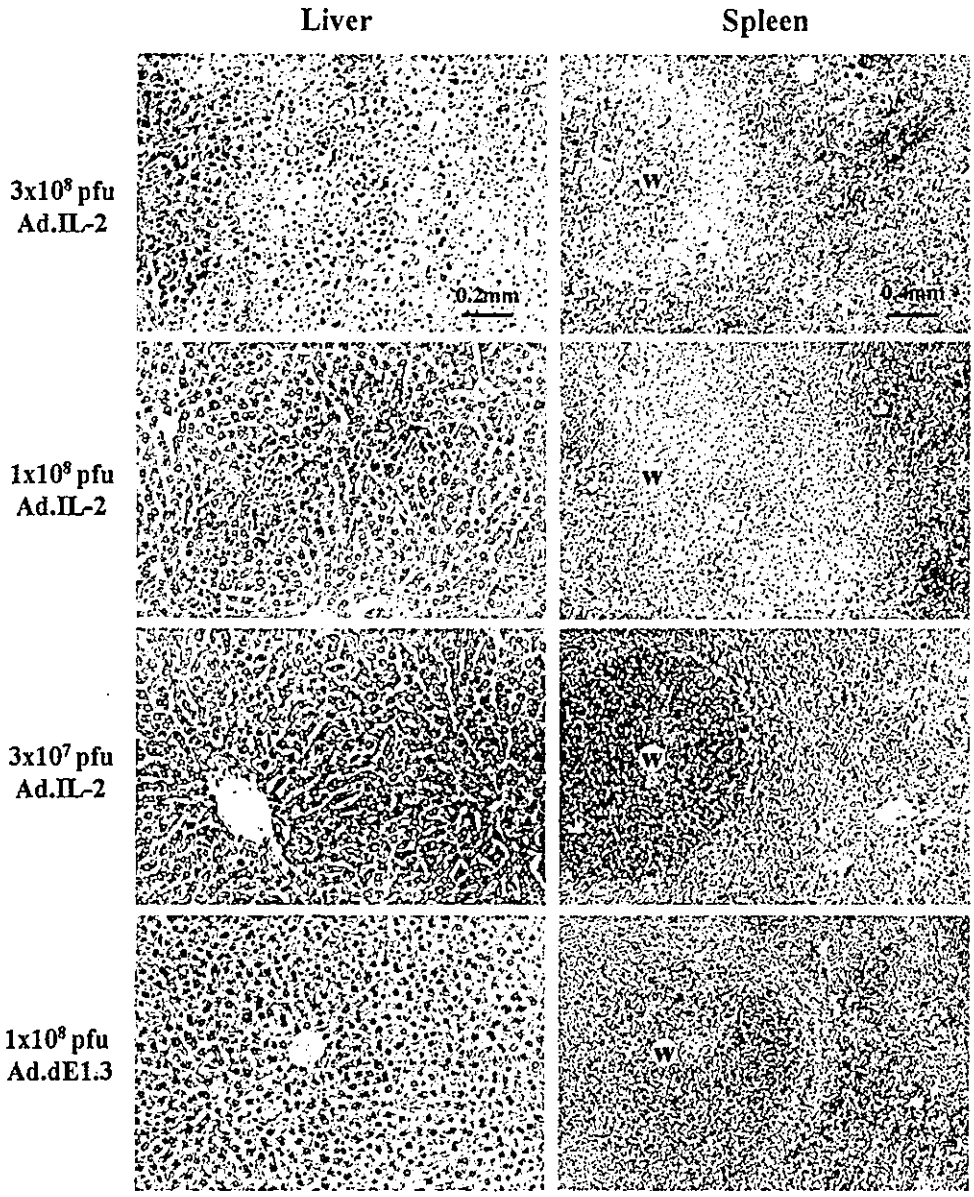

Figure 6. Representative histology pictures of liver and spleen at 3 days after an injection of Ad.IL-2 or Ad.dE1.3 into the primary tumor. All specimens were stained with H&E. White pulp (w) in the spleen was severely deteriorated in mice treated with $3x10^8$ or $1x10^8$ pfu of Ad.IL-2. Original magnification x200 (liver) and x100 (spleen).

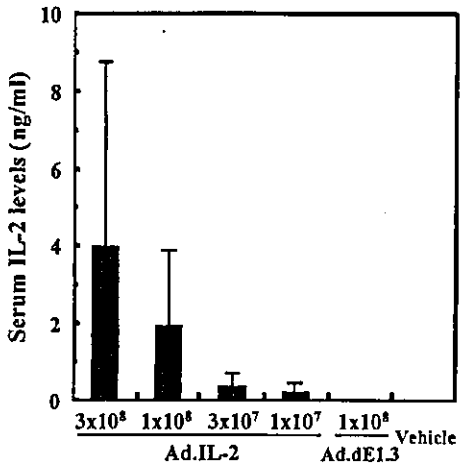

Figure 7. IL-2 levels in serum. Blood was collected at 3 days after an injection of each Ad into the primary tumor, and IL-2 levels in serum were measured by ELISA.

on the primary tumor is beneficial, the more substantial role of the primary tumor in the present strategy is as the target site where Ad encoding cytokine gene is feasibly accessed and where it can exert a systemic antitumoral immune reaction efficiently, leading to subsequent eradication of micro-metastasis in distant organs. Such a clear concept has not yet emerged, and forms a clinical viewpoint, this is quite unique and meaningful.

The second concept and of greatest importance here is our novel finding of the optimal therapeutic expression level of IL-2 (i.e., not the highest one) needed for successful IL-2 gene therapy in terms of not only minimizing side effects but also eliciting the maximal therapeutic effect. It is likely that the vector dose administered previously was determined based only on the desire to cause minimal side effects and not from the viewpoint of obtaining the optimal cytokine level to confer antitumoral immunity most effectively. One might ingenuously recommend an increased

vector dose encoding the cytokine gene to the toleration level with respect to side effects in animals or in human patients. The present study clearly showed that only the optimal therapeutic expression level of IL-2 could induce the systemic antitumoral immunity most effectively and reveal the maximal therapeutic effects.

In addition, both the liver and spleen were minimally or severely damaged when the optimal or more than the optimal dose of Ad.IL-2 was used, respectively. Immunological mechanisms of IL-2-induced antitumoral immune reaction, with which the present pathological findings are consistent, have been well elucidated and are not the focus or the aim of the present study. We have already shown that $CD4^+$ and $CD8^+$ T cells and macrophages and/or natural killer cells mainly infiltrate tumor lesions and that tumor-specific $CD8^+$ cytotoxic T cells especially play an important role in eliciting an antitumoral immune reaction after IL-2 gene therapy alone or in combination with suicide gene therapy (1,2,4). We, rather, chose IL-2 as a therapeutic gene in the present study because immunological mechanisms of IL-2 have been extensively investigated and elucidated. Despite extensive immunological analyses, actual clinical outcomes have led to unsatisfactory results, which is due to insufficient analyses and assessments from the clinical viewpoint. In this regard, more important and substantial finding in the present study is the injury in splenic white pulp, which is normally composed of B and T lymphocytes and other immune effector cells. This may be due to the disorganization of these immune effector cells by too high a concentration of circulating IL-2, because the injury to the spleen was not apparent when the optimal dose of Ad.IL-2 that elicited the maximal therapeutic effect was used. Thus, the present pathological findings indicate that the spleen may play an important role in IL-2 gene therapy and spleen injuries may result in the diminished therapeutic effect seen with the higher than optimal dose of Ad.IL-2. Moreover, we found that liver and spleen injuries were recoverable, and, notably, that severe damage in the spleen was almost normalized within a month. These findings are encouraging with regard to the potential safety and clinical applicability of this approach. On the other hand, the narrow range of the optimal therapeutic expression level of IL-2, i.e., only a single available dose in the present model, may be a critical issue and further emphasize the importance for successful clinical application. In this regard, we demonstrated that serum IL-2 level may be a good indicator for determining the optimal therapeutic expression level and monitoring the risk of systemic side effects, because it was consistently parallel to the administered doses of Ad.IL-2 into the primary tumor lesion and because it can be feasibly measured in human patients as well.

The third novel concept is that local and predominant secretion of a cytokine from connective tissues adjacent to a tumor instead of the tumor itself can elicit sufficient antitumoral immunity, which indicates that cytokine gene therapy does not require high gene transduction efficiency in the tumor itself, in contrast to previously held beliefs. It has been believed that inefficient gene transduction efficiency in tumors may largely limit the clinical utility of cancer gene therapy in general (31,32), and some investigators have tried to increase or target gene transduction efficiency in the tumor itself even in the case of cytokine gene therapy (33). Obviously, the fact that any current vector cannot transduce a therapeutic gene to every cancer cell by *in vivo* administration has substantially limited the clinical utility and benefit of gene transfer of intracellular molecules (34). In fact, Ad-mediated suicide gene therapy, which exhibits the potent bystander effects for a variety of cancers (19,35-37), did not show apparent regression of primary or metastatic tumors in the present animal model (data not shown). In contrast, we clearly demonstrated that an injection of Ad.IL-2 into the primary tumor, in which gene transduction efficiencies *in vitro* and *in vivo* were extremely low, could have a sufficient therapeutic effect, as shown by the fact that local secretion of IL-2 from the connective tissue adjacent to the tumor induced a systemic antitumoral immune reaction. Because the role of gene transduction efficiency in the tumor in cytokine gene therapy has been to date overestimated or uncertain, this novel concept and contradictory finding may largely facilitate the clinical use of cytokine gene therapy and may be important for planning and developing future strategies.

Taken together, from the clinical viewpoint, all of the properties of the present strategy may allow for its clinical use for osteosarcoma patients. It is feasible that an injection of Ad.IL-2 into the primary lesion may be commonly administered to osteosarcoma patients before conventional treatments, regardless of the pre-existence, or lack thereof, of lung metastasis, as long as side effects and serum IL-2 levels are carefully monitored. Any therapeutic effects against lung metastasis, which may result from both prophylactic treatment via a cancer vaccine for sequential metastasis and regression of tumor growth of pre-existent metastasis, would be beneficial for osteosarcoma patients, even though Ad.IL-2 gene therapy alone cannot completely cure them.

In conclusion, just a single injection of the optimal dose of Ad.IL-2, presumably Ad encoding some cytokine genes as well, only into the primary lesion may be clinically useful in terms of feasibility, low invasiveness, cost effectiveness as well as therapeutic potential for disseminated metastatic cancers. More generally and importantly, we have revealed novel concepts that have been missing but crucially necessary for successful cytokine gene therapy, that is, the optimal therapeutic expression levels of a cytokine, the fact that high gene transduction efficiency in the tumor itself is unnecessary and the strategy of treating only the primary tumor in order to eradicate metastatic tumors without any direct treatment to the latter.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (C) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, the Nakatomi Foundation and a Grant from Japan Orthopaedics and Traumatology Foundation, Inc. (No. 0099 and No. 0130). We thank M. Uenosono for her technical assistance, Dr E. Mekada for helpful comments, Dr H. Hamada for providing Ad.IL-2 and Dr H. Yoshikawa and Dr A. Uchida for providing LM8 cells.