Under immunofluorescence microscopy, mixed glial cell cultures infected with CZPG for 3 days showed double-positive cells, i.e., β-Gal+/GFAP+ cells (infected astrocyte) and β-Gal+/GC+ cells (infected oligodendrocytes), indicating that both astrocytes and oligodendrocytes were successfully transduced to express β-Gal encoded by CZPG (Fig. 2). Of 192 GFAP+cells, 20 were positive for β-Gal (transduction efficiency; 10.4%). Of the 202 GC+ cells, 72 (35%) were positive for β-Gal. These results indicate that CZPG can transduce oligodendrocytes more efficiently than astrocytes in primary culture of adult rat brain (Fig. 3).

2. Efficient transduction of neuronal cells in vivo by CZPG

To test the ability of CZPG to infect glial cells in vivo, we injected CZPG into the mouse striatum (n= 12). Three days after injection, β-Gal positive cells were demonstrated in the parenchyma (Fig. 4 P1, P2, P3) and periventricular zone (Fig. 4 V1, V2, V3). We detected transduction mainly for parenchymal cells (P1, P2, P3) and ependymal cells (V1, V2), partially migrated into the parenchyma (V3). Next, we tried immunohistochemistry *in vivo*, double-positive cells, i.e., β-Gal+/GFAP+ cells (infected astrocytes) and β-Gal+/CNP+ cells (infected oligodendrocytes) were demonstrated by double immunofluorescence microscopy, indicating that both astrocytes and oligodendrocytes can also be transduced *in vivo* by CZPG (Fig. 5). We observed no cytological damage *in vitro* and no histological evidence of cell damage, inflammation, or cell infiltration after infection with CZPG.

DISCUSSION

Gene therapy for neurological disorders requires an efficient and stable gene delivery system for the CNS. As for gene transfer systems into the CNS cells, adenovirus, adeno-associated virus (AAV), herpesvirus, and lentivirus vectors have been studied[17]. Although adenovirus vector has been reported to be a useful gene transfer system for the nervous system[18-20], its expression is transient because the vector does not integrate into the host genome. Moreover, first-generation adenovirus vectors cause severe tissue inflammation when inoculated in brain tissue[21]. The adeno-associated virus-mediated gene transfer system is promising because no cellular toxicity has been reported and long-term expression by integration has been reported[20,22,23]. However, a problem with this system is the difficulty of preparing a sufficiently large virus stock for clinical use. Herpes simplex virus-based vectors have particular advantages for gene delivery into the nervous system including their ability to infect nondividing neurons and long-term expression. Disadvantages of this system are host immune responses and inflammatory and toxic reactions[24,25]. Some investigators have reported that lentivirus vector is promising for the transfer of genes into CNS cells, although its safety remains unclear[26,27].

To seek an alternative approach to overcome the limitations of current vector systems for transducing CNS cells, we tested a baculovirusmediated gene transfer system in rodent CNS cells in vivo and in vitro. The generation of recombinant baculovirus is relatively less time-consuming, and expansion of the virus stock is relatively easy. The titer of the virus stock after purification is high and comparable to that of adenovirus. Large DNA (up to 15 kb) can be inserted into the transfer vector. Moreover, baculovirus cannot replicate in mammalian cells, and no cell toxicity has been observed[28]. In fact, there was no evidence of tissue inflammation or cell damage in the present study.

The first attempt to transfer genes to mammalian cells with the baculovirus vector was reported by Hofman et al. in 1995. They successfully transferred a gene to mammalian hepatocytes. However, they failed to transfer genes to neural cell lines, such as mouse neuroblastoma Neuro-2a and human astrocytoma SW 1088[2]. Recently, Sarkis et al. have reported successful transduction of nervous system cells (both neurons and astrocytes) *in vitro* and *in vivo* using nonpseudotype baculovirus[5]. In the present study, we used a pseudotype vector. By pseudotyping, the VSVG protein is expressed in the viral envelope and mediates the escape of the recom-

binant pseudotype baculovirus from the intracellular vesicles. The efficiency of the escape may be the rate-limiting step for transduction[6]. We also tried a nonpseudotype baculoviral vector and detected efficient transduction *in vitro*, especially in the liver cell line Hep G2, but detected no transduction *in vivo* (data not shown). The baculovirus vector CZPG prepared in this study could transduce both astrocytes and oligodendrocytes. This ability implies a potential advantage of pseudotype baculovirus vectors for use in CNS gene therapy, because oligodendrocytes are the cells principally affected in many neurological diseases, such as globoid cell leukodystrophy (Krabbe's disease), metachromatic leukodystrophy, and adrenoleukodystrophy. We are now generating a baculovirus vector expressing galactocerebrosidase, which is lacking in Krabbe's disease, to investigate its therapeutic effects in a mouse model of Krabbe's disease. Our failure to find cytological or histological evidence of cell damage, inflammation, or cell infiltration after infection of CZPG *in vitro* and *in vivo* point is also important for gene transfer into CNS and other organ systems *in vivo*.

There are several unanswered questions regarding our baculovirus vector system; i.e., how long the expression persists and how efficiently it infects oligodendrocytes *in vivo*. For the treatment of Krabbe's disease, highly efficient transduction of oligodendrocytes and persistent expression of transferred genes will be required. Additional experiments are underway to answer these questions.

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Adenovirus Vector-Mediated Gene Transfer Using Degradable Starch Microspheres for Hepatocellular Carcinoma in Rats

Hiroaki Shiba, M.D., Ph.D.,*,†,[1] Tomoyoshi Okamoto, M.D., Ph.D.,* Yasuro Futagawa, M.D., Ph.D.,* Takeyuki Misawa, M.D., Ph.D.,* Katsuhiko Yanaga, M.D., Ph.D.,* Toya Ohashi, M.D., Ph.D.,† and Yoshikatsu Eto, M.D., Ph.D.†

*Department of Surgery and †Department of Gene Therapy, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan

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Background. **When gene therapy is performed for malignant tumors, gene transfer efficiency and selectivity are extremely important. The delivery of anticancer agents and embolic agents through tumor feeding artery is known as transarterial embolization. We speculated that genes might be efficiently and selectively transferred to hepatocellular carcinomas (HCCs) by degradable starch microspheres (DSM) as the embolic agent, which could be trapped within the tumor and release a gene vector. Therefore, we studied the use of DSM for adenovirus vector-mediated gene transfer to HCC *in vivo*.**

Material and methods. **HCC was induced in rats with diethylnitrosamine and phenobarbital, after which either AxCALacZ and DSM or AxCALacZ alone was injected through the hepatic artery.**

Results. **Histological examination revealed that β-galactosidase expression was greater ($P < 0.001$), and more selective ($P < 0.001$) in tumors after injection of AxCALacZ and DSM, than after injection of the vector alone.**

Conclusion. **Injection of DSM together with an adenovirus vector through the hepatic artery can result in efficient and cancer-selective transfer of genes to HCC.**

Key Words: **gene transfer; hepatocellular carcinoma; adenovirus vector; arterial injection; degradable starch microspheres; rat.**

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world [1, 2], for which current treatments other than liver transplantation for nonadvanced HCC are regarded as palliative. Therefore, new therapeutic modality should be developed to further improve the outcomes of HCC. Thus far, effective gene therapy has not been reported for HCC because the efficiency and selectivity of gene transfer to the tumor tissue are too low [3, 4]. Unlike normal hepatocytes that receive dual blood supply from the hepatic artery and the portal vein, nonearly HCC receives their blood supply exclusively from the hepatic artery [2, 5]. On the basis of such a peculiar feature of HCC, the efficiency and tumor selectivity of anticancer agents can be increased by injection of such agents with embolic material through the hepatic artery, a technique known as transarterial embolization (TAE) [6–8]. When embolic material also is injected through the feeding artery, the anticancer agents remain within the tumor vessels and exert a stronger and more selective antitumor effect. Therefore, injection of an embolic agent with an adenovirus vector may be useful for achieving efficient and selective gene transfer to HCC. In fact, we have already reported that gene transfer efficiency and selectivity are increased by intra-arterial injection of an adenovirus vector with iodized oil ester, an embolic agent [9].

Degradable starch microspheres (DSMs) are another embolic agent used in transarterial chemotherapy for HCC and metastatic liver tumors. DSMs are degraded by serum amylase and become progressively smaller, with the $t_{1/2}$ for complete dissolution being between 15

[1] To whom correspondence and reprint requests should be addressed at Hiroaki Shiba, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan. E-mail: hs0817@ss.iij4u.or.jp.

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and 30 min *in vitro* [10]. Because of this short half-life, DSM allows repeated treatment through the hepatic artery.

We hypothesized that injection of an adenovirus vector with DSM into the feeding artery might cause the vector to reside longer within HCC and improve the efficiency of gene delivery to the tumor, while preventing delivery of the gene to non-tumor tissues. The purpose of this study was to evaluate the efficiency and cancer selectivity of gene transfer by adenovirus vector plus DSM [11, 12].

MATERIALS AND METHODS

Microspheres

DSMs (Spherex®, Pharmacia, Uppsala, Sweden) were obtained through Yakult Co. Ltd. (Tokyo, Japan). They were cross-linked starch microspheres measuring 45 ± 7 μm in diameter. DSMs were stored at room temperature in the dark until use.

Recombinant Adenovirus Vector

The replication-defective recombinant adenovirus vector (AxCALacZ) expressing the *Escherichia coli* β-galactosidase (β-gal) was a kind gift from Dr. Saito (Institute of Medical Science, University of Tokyo, Tokyo, Japan) [13, 14]. The vector was generated from adenovirus type 5, and the E1 and E3 regions were deleted to prevent viral replication. The β-gal gene was driven by the cytomegalovirus-enhancer-chicken β-actin hybrid promoter (CAG promoter) [15] and a rabbit beta-globin poly (A) signal located downstream from the gene. The vector was purified by two rounds of CsCl centrifugation and then was stored at −80°C until use. In this study, we used the vector at a concentration [16] of 2.0×10^8–2.0×10^{10} pfu/mL.

Rat HCC Model

Male Wistar rats (6 weeks old and 180 g) were housed in plastic cages with shredded paper bedding (Alpha-dri, Shepherd Specialty Papers Inc., Kalamazoo, MI) in a biological cabinet at the Laboratory Animal Facility of Jikei University School of Medicine. The animals were maintained with a 12-h light–dark cycle at a temperature of 22 ± 2°C and 55 ± 5% humidity in a room with a filtered air supply. An acclimatization period of 4 days was set. The weight of the rats was recorded every week. The rats received 10 mg/kg per day of diethylnitrosamine (DENA; Sigma Chemical Co., St. Louis, MO) and 50 mg/kg per day of phenobarbital (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in drinking water for 16 weeks. At the end of the induction period, five animals were sacrificed to check for tumor formation. Tumors were only found in the liver. There were multiple hepatic tumor nodules in each of the five animals, which ranged from 1 to 10 mm in diameter. Gene transfer efficiency and cancer selectivity were studied in the animals in which developed HCC. It was previously reported that HCC induced in rats by DENA and phenobarbital is hypervascular and is supplied by a tumor-feeding artery [11, 12]. The protocol for this animal study was approved by the Laboratory Animal Facility of Jikei University School of Medicine and was performed in accordance with the National Institutes of Health Guidelines.

Assessment of Gene Transfer to HCC

The animals were anesthetized with pentobarbital (Nembutal®, Abbott Laboratories, North Chicago, IL). After clamping the gastroduodenal artery, either 5.0×10^7 pfu/250 μL of AxCALacZ (n = 15) or 5.0×10^7 pfu/100 μL of AxCALacZ plus 150 μL of DSM (n = 5) was injected through the hepatic artery [17] into the liver using a 30-gauge needle and a 1-mL syringe. After injection, the hepatic artery was compressed to stop any bleeding. Two days later, the animals were sacrificed and specimens were obtained from including the tumor and from the non-tumor tissue in different regions. The specimens were placed in O.C.T. compound (Tissue-Tek®, Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen in liquid nitrogen. A section (8-μm thick) was cut from each specimen and fixed in 0.25% glutaraldehyde buffered in PBS (pH 7.0) for 10 min at 4°C. After fixation, the sections were incubated in X-gal (5-brom-4chlor-3-indolyl-β-D-galacto-pyranosid) staining solution (2 mM $MgCl_2$, 4 mM K3FeCN, 4 mM K4FeCN, and 0.4 mg/mL X-gal in PBS, pH 7.0) [18] for 12 h at 37°C and then counterstained with hematoxylin-eosin.

Histological Examination

For assessment of the efficiency and selectivity of gene transfer by intra-arterial injection of AxCALacZ with or without DSM, we examined β-gal expression in tumor and nontumor tissue by detecting blue X-gal staining using NIH Image (National Institutes of Health, Bethesda, MD). The percentage of blue-stained tumor tissue was calculated as the blue area of the tumor divided by the total area of the tumor. The percentage of blue-stained nontumor tissue was calculated similarly. Then, the percentage of blue area of the tumor was used to assess gene transfer efficiency, whereas the percentage of blue area of the tumor/percentage of blue area of nontumor tissue was calculated for the assessment of selectivity.

Statistics

The nonparametric Mann-Whitney's t test was used for statistical analysis. The results were expressed as a mean ± standard deviation (SD), and probability values of less than 0.05 were considered statistically significant. Analyses were done using a Stat View software.

RESULTS

Gene Transfer to HCC

We injected AxCALacZ with or without DSM into the hepatic artery so as to reach the tumors of rats with HCC, and then examined β-gal expression in tumor and nontumor tissue using X-gal staining to detect β-gal expression as blue areas. After injection of the AxCALacZ vector alone, little β-gal expression was detected in tumors, and expression was more extensive in the surrounding nontumor tissue (Fig. 1A). In contrast, when AxCALacZ plus DSM were injected into the hepatic artery, a high level of β-gal expression was detected in tumors, while little β-gal expression was found in the surrounding non-tumor tissue (Fig. 1B). These results suggested that intra-arterial injection of an adenovirus vector with DSM could efficiently and selectively deliver a gene to HCC.

Histological Findings

For assessment of the efficiency and selectivity of gene transfer by AxCALacZ with or without DSM, we measured β-gal expression in tumor and nontumor tissue using NIH Image and compared the results obtained by the two methods. First, we assessed the efficiency of gene transfer to tumors by the two methods.

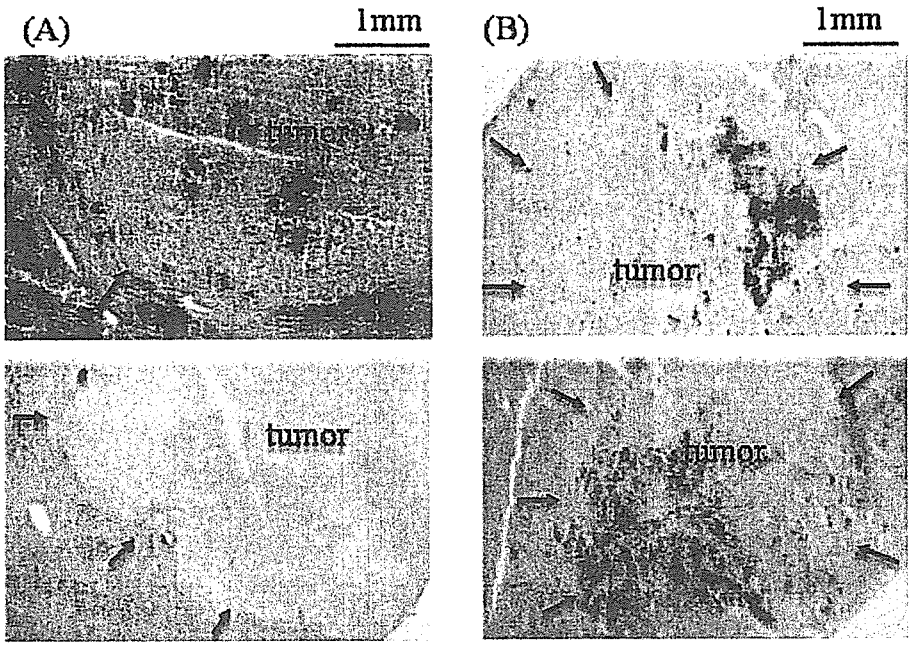

FIG. 1. β-gal expression in the livers. Animals with HCC were injected with either 5.0×10^7 pfu/250 μL of AxCALacZ ($n = 15$) or 5.0×10^7 pfu/100 μL of AxCALacZ and 150 μL of DSM ($n = 5$) through the hepatic artery and were sacrificed 2 days after the injection. The livers were frozen, sectioned, and stained with X-gal and hematoxylin-eosin. (A) The liver after injection of AxCALacZ alone; little β-gal expression was detected in tumors, and expression was more extensive in the surrounding non-tumor tissue. (B) The liver after injection of AxCALacZ and DSM; a high level of β-gal expression was detected in tumors, whereas little β-gal expression was found in the surrounding non-tumor tissue.

In tumors, AxCALacZ plus DSM ($n = 26$) achieved a higher level of β-gal expression (23.2 ± 20.4%) than AxCALacZ alone ($n = 40$; 8.7 ± 11.3%; Fig. 2A). Then, we assessed the selectivity of each treatment. When AxCALacZ alone was injected, β-gal expression in tumors was 1.4 ± 2.0 times higher than that in the surrounding non-tumor tissue, whereas injection of AxCALacZ and DSM increased β-gal expression to 5.9 ± 5.3 times the level in the surrounding tissue (Fig. 2B). These results suggest that intra-arterial injection of an adenovirus vector plus DSM can increase the efficiency and selectivity of gene transfer to HCC.

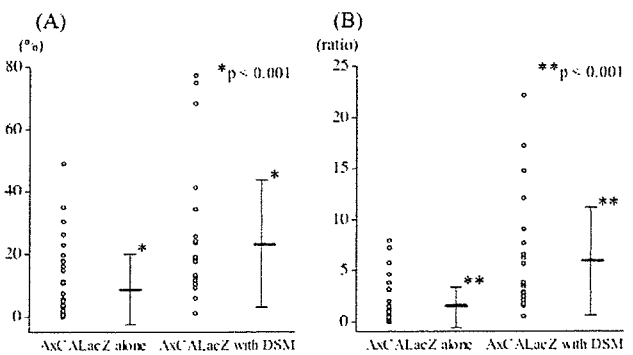

FIG. 2. Image analysis of photomicrographs. The extent of β-gal expression in tumor and nontumor tissue was measured using NIH Image software. The percentage blue area of the tumor was used to assess gene transfer efficiency, whereas the percentage blue area of tumor tissue/percentage blue area of nontumor tissue was calculated to assess selectivity. (A) the percentage blue area in tumors and mean ± SD; (B) the percentage blue area of the tumor/percentage blue area of nontumor tissue in tumors and mean ± SD.

DISCUSSION

In gene therapy for malignant tumors, highly efficient gene transfer to the tumor and minimization of transfer to normal tissues are extremely important. We have already reported the feasibility of an oily contrast medium as an embolic material for satisfactory gene transfer to liver cancer [9]. It seems likely that the prolonged residence of the adenovirus vector within the tumor vessels by the embolic agent increases contact between the vector and tumor cells, resulting in enhanced gene expression within the tumor and limiting gene transfer to the surrounding normal tissues.

In our preliminary study in vitro, β-gal was more extensively expressed in rat metastatic liver tumor cell line during 48 to 72 h after transfection of AxCALacZ with or without DSM, and there was no significant difference in each treatment group (data not shown). As the interval between transfection and sacrifice, we chose 2 days, following the example of previous reports on gene transfer for HCC using adenovirus vector [19, 20].

DSM are tiny embolic spheres made from starch. Because DSMs are digested by serum amylase, the half-life is short and, therefore, the injection of microspheres does not affect portal blood flow, thus giving the advantage of allowing repeated administration [10]. In a recent report, repeated hepatic arterial infusion of viral vector was useful for gene therapy for HCC [21]. We believe that repeated co-administration of DSM with a viral vector via the hepatic artery has potential for improving the efficacy and cancer-selectivity of gene transfer to HCC. In this study, we coadministered DSM and adenovirus vector through the hepatic artery to HCC in rats and achieved a high gene transfer efficiency as well as selectivity delivering to tumors (Figs. 1 and 2). However, the extent of gene transfer i.e., 23.2% of the tumor cells being infected, still seems less than optimal. Because the viral vector/DSM ratio and the amount of DSM per unit tumor diameter can be manipulated, modification of such variables may further improve the efficacy of gene transfer.

Because DSM also are used as an embolic agent in intra-arterial chemotherapy for metastatic liver tumors and because the coadministration of DSM with an adenovirus vector via the hepatic artery may achieve a higher gene transfer efficiency and tumor selectivity in gene therapy for metastatic liver tumors, further optimization of this method seems necessary. In conclusion, combined adenovirus vector and DSM, a common tumor embolic agent, enables efficient gene transfer with a high tumor selectivity to HCC in rats.

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ORIGINAL REPORT

Multicenter Phase I Study of Repeated Intratumoral Delivery of Adenoviral *p53* in Patients With Advanced Non–Small-Cell Lung Cancer

Toshiyoshi Fujiwara, Noriaki Tanaka, Susumu Kanazawa, Shoichiro Ohtani, Yasuo Saijo, Toshihiro Nukiwa, Kunihiko Yoshimura, Tetsuo Sato, Yoshikatsu Eto, Sunil Chada, Haruhiko Nakamura, and Harubumi Kato

From the Center for Gene and Cell Therapy, Okayama University Hospital; Departments of Surgery and Radiology, Okayama University Graduate School of Medicine and Dentistry, Okayama; Department of Molecular Medicine, Tohoku University Graduate School of Medicine; Department of Respiratory Oncology and Molecular Medicine, Institute of Developing, Aging, and Cancer, Tohoku University, Sendai; Department of Gene Therapy, Institute of DNA Medicine, Department of Respiratory Medicine, Jikei University School of Medicine; Department of Surgery, Tokyo Medical University, Toyko, Japan; and Introgen Therapeutics Inc, Houston, TX.

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

Authors' disclosures of potential conflicts of interest and author contributions are found at the end of this article.

Address reprint requests to Toshiyoshi Fujiwara, MD, Center for Gene and Cell Therapy, Okayama University Hospital, 2-5-1 Shikata-cho, Okayama 700-8558, Japan; e-mail: toshi_f@md.okayama-u.ac.jp.

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ABSTRACT

Purpose
To determine the feasibility, safety, humoral immune response, and biologic activity of multiple intratumoral injections of Ad5CMV-p53, and to characterize the pharmacokinetics of Ad5CMV-p53 in patients with advanced non–small-cell lung cancer (NSCLC).

Patients and Methods
Fifteen patients with histologically confirmed NSCLC and *p53* mutations were enrolled onto this phase I trial. Nine patients received escalating dose levels of Ad5CMV-p53 (1×10^9 to 1×10^{11} plaque-forming units) as monotherapy once every 4 weeks. Six patients were treated on a 28-day schedule with Ad5CMV-p53 in combination with intravenous administration of cisplatin (80 mg/m^2). Patients were monitored for toxicity, vector distribution, antibody formation, and tumor response.

Results
Fifteen patients received a total of 63 intratumoral injections of Ad5CMV-p53 without dose-limiting toxicity. The most common treatment-related toxicity was a transient fever. Specific *p53* transgene expression was detected using reverse-transcriptase polymerase chain reaction in biopsied tumor tissues throughout the period of treatment despite of the presence of neutralizing antiadenovirus antibody. Distribution studies revealed that the vector was detected in the gargle and plasma, but rarely in the urine. Thirteen of 15 patients were assessable for efficacy; one patient had a partial response (squamous cell carcinoma at the carina), 10 patients had stable disease, with three lasting at least 9 months, and two patients had progressive disease.

Conclusion
Multiple courses of intratumoral Ad5CMV-p53 injection alone or in combination with intravenous administration of cisplatin were feasible and well tolerated in advanced NSCLC patients, and appeared to provide clinical benefit.

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INTRODUCTION

Lung cancer is the most common cause of cancer related deaths in both men and women worldwide.[1] In 2001, 39,880 males and 15,122 females died of lung cancer in Japan, which ranked first among males and third among females in the number of cancer deaths.[2] Recent advances in molecular biology have fostered remarkable insights into the molecular basis of lung cancer,[3] and suggest that restoration of the function of critical gene products could halt or reverse cancer pathogenesis, thus having a therapeutic effect in cancer.

p53 is the most extensively studied tumor suppressor gene, and its mutation has been reported to be one of the most common genetic changes found in malignant tumors.[4] *p53* gene mutation is reported to occur in 40% to 50% of non–small-cell lung cancer (NSCLC),[5] and aberrant *p53* expression correlates with an adverse prognosis in lung cancers.[6,7] The *p53* gene product is involved in multiple pivotal cellular processes as a potent transcriptional regulator, and one of its most important roles is in the regulation of apoptosis.[8] We previously reported that the overexpression of the wild-type *p53* (wt-*p53*) gene by recombinant, replication-deficient viral vector, Ad5CMV-p53 (ADVEXIN; Introgen Therapeutics Inc, Houston, TX), triggered apoptosis in a variety of human cancer cells independent of their *p53* status.[9-13] ADVEXIN in combination with chemotherapeutic drugs, such as cisplatin, showed a profound antitumor effect in

vitro and in vivo.[14,15] We also found that wt-*p53* gene transfer could promote bystander effects to neighboring tumor cells through the multiple mechanisms, including antiangiogenesis and neutrophil-mediated immune responses.[16,17]

On the basis of these promising preclinical results, a multi-institutional, dose-escalation phase I study of ADVEXIN was conducted in Japanese patients with advanced NSCLC who had failed conventional treatments such as chemotherapy and radiotherapy. We assessed the tolerability of repeated administration of ADVEXIN for more than 6 months, which was not examined in previous phase I and II trials in the United States.[18-20]

PATIENTS AND METHODS

Patient Eligibility

Patients were included who met the following criteria: between 15 and 75 years old; histologically confirmed advanced NSCLC, resistant or refractory to standard therapies; lesions accessible for repeated injection; measurable disease; Zubrod performance status of 2 or lower; life expectancy greater than 12 weeks; and adequate bone marrow, liver, and renal function. Pretreatment tumor biopsies must show a *p53* mutation by single-strand conformation polymorphism analysis.

Study Design

The protocol was approved by the institutional review board of the participating institutions as well as by the Ministry of Health and Welfare. The trial was a multicenter, open-label, phase I dose-escalation study of ADVEXIN. Six patients were treated per dose level, starting with a dose of 1×10^9 plaque-forming units (PFU; 2×10^{10} virus particles) and escalating in log increments to a maximal dose of 1×10^{11} PFU (2×10^{12} virus particles). Initially, three patients were treated with ADVEXIN as monotherapy at each dose level. If no toxicity was observed with the vector, then the next three patients received ADVEXIN in combination with intravenous administration of cisplatin. In a cohort of 1×10^{11} PFU, only three patients were treated with ADVEXIN alone because of the modification of the protocol. As many new drugs including taxanes, irinotecan, and vinorelbine became available in Japan during this trial, and cisplatin was no more a sole key drug for NSCLCs, the protocol was modified.

Treatment Plan

The construction and generation of ADVEXIN was reported previously.[9,14,21] The vector was injected directly into the primary tumor, either endobronchially using a bronchoscope or percutaneously under computed tomography (CT) guidance. For lesions of at least 4 cm in the largest diameter, the final volume administered was 10 mL; for lesions with a diameter of less than 4 cm, the final volume injected was 3 mL. In the cohorts with ADVEXIN plus cisplatin, patients were treated intravenously with 80 mg/m^2 of cisplatin on day 1 and study vector injected intratumorally on day 4. Treatments were repeated every 28 days.

Toxicity and Response Criteria

Patients were monitored with regard to safety and tolerability according to the National Cancer Institute's Common Toxicity Criteria (version 2) for a minimum of 12 months or until death. Indicator lesions were measured serially by bronchoscope or radiographic scanning, and underwent biopsy for histology and molecular analyses. Standard response criteria were used to evaluate target tumor response.

Determinations of Antibody Titer

Serum samples were collected at baseline and before each course of treatment, and tested for the presence of neutralizing antiadenovirus antibody and anti-p53 antibody. Neutralizing antibodies to Ad5 were detected by their ability to inhibit the cytopathic effect (CPE) of adenovirus on 293 cells. The mixture of an Ad5 suspension and diluted plasma was applied to a monolayer of 293 cells in 96 well plates. The cells were incubated until the appearance of CPE. The titer of neutralizing antibodies in plasma was equivalent to the inverse value of the dilution at which 100% of the CPE was observed. The titers of anti-p53 antibodies were tested by means of a qualitative enzyme-linked immunosorbent assay kit.

Analysis of Tumor Biopsy Tissues

Pretreatment (immediately before) and post-treatment (48 hours after) tumor biopsy specimens were obtained by core biopsies of the vector-injected tumor after each course of treatment. Tissue samples were also used for conventional reverse-transcriptase polymerase chain reaction (RT-PCR) assay to detect expression of exogenous p53. Total RNA was isolated from frozen biopsy samples and used as a template. After RT, a nested PCR procedure was used, with vector-specific primers. mRNA copy numbers of exogenous p53, p21, Noxa, p53AIP1, and *β-Globin*, the housekeeping gene, were also determined by real-time quantitative RT-PCR using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany), a LightCycler DNA Master SYBR Green I kit (Roche Diagnostics), and LightCycler-Control Kit DNA (Roche Diagnostics). All expression levels were normalized to that of β-Globin.

Analysis of Vector Dissemination and Biodistribution

ADVEXIN shedding was monitored in the gargle, urine, and plasma specimens by a vector-specific DNA-PCR assay or the CPE assay. DNA was isolated from samples and analyzed for the presence of vector DNA by PCR. The vector-specific primers were used to detect p53 open-reading-frame/adenoviral DNA junction. The CPE assay is a bioassay to semiquantitatively detect the amount of vector contained in a biologic fluid.

RESULTS

Patient Characteristics

Fifteen patients with advanced NSCLC who had failed prior conventional therapies were enrolled onto this clinical trial from February 1999 to April 2003 (Table 1). Patients were primarily male (14 males and 1 female), with median age of 58 years (range, 46 to 71 years). Nine patients (60%) had prior chemoradiotherapy and six patients (40%) had prior chemotherapy. Fourteen patients (93.3%) were treated with platinum-based regimens containing either cisplatin or carboplatin. The median number of courses per patient was three (average, 4.2), and the range was one to 14 courses.

Adverse Events

Toxicities and adverse events (AE) that occurred in all patients are presented in Table 2. Among the 15 treated patients, none withdrew from the study as a result of toxicity, and no grade 4 toxicities classified as being possibly, probably, or definitely related to the vector were observed. The most frequent vector-related AE was a transient fever. In most patients (93.3%), self-limited fever developed 6 to 24 hours after ADVEXIN injection regardless of dose, with a highest reported grade of 3. Patients generally recovered within 48 hours, although four patients (26.7%; patients 3, 7, 8, and 13) developed grade 2 or 3 obstructive pneumonia categorized as not related or probably not related to study medication. Hematologic toxicity was limited, with one incidence of grade 1 leukopenia (6.7%) and three incidences of grade 2 or 3 anemia (20%). Transient, mild increases in liver ALT and AST were noted in one patient treated with ADVEXIN alone. Liver enzymes recovered to pretreatment values within 10 days.

Clinical Outcome

Thirteen of 15 patients (86.7%) were assessable for response with CT scan, broncoscopic, and clinical findings. Objective responses were a partial response in one patient (7.7%), stable disease (SD) in 10 patients (76.9%), and progressive disease in two patients (15.4%; Table 1). The median time of SD was 4.4 months (range, 1 to 11

Table 1. Characteristics of Patients and Tumors Who Received Injections of ADVEXIN,* and Response of Injected Lesion

Patient No.	Sex	Age (years)	Histology	Location of Tumor	Prior Therapy	TNM Stage	Method of Injection	Viral Dose ± Cisplatin	No. of Courses	Response
1	Male	58	Squamous	Carina	Chemotherapy; radiotherapy	cT4N0M0 stage IIIB	Bronch	10^9 PFU	14	PR
2	Male	58	Squamous	Left lower lobe	Chemotherapy; radiotherapy	cT4N2M0 stage IIIB	Bronch; CT	10^9 PFU	9	SD
3	Male	66	Squamous	Right mainstem bronchus	Surgery, laser; chemotherapy	cT2N0M0 stage IB; (pre-operative)	Bronch	10^9 PFU	4	PD
4	Female	46	Adeno	Left upper lobe	Chemotherapy	cT2N3M1 stage IV	CT	10^9 PFU + cisplatin	10	SD
5	Male	55	Squamous	Right lower lobe	Chemotherapy	cT4N1M0 stage IIIB	Bronch; CT	10^9 PFU + cisplatin	3	SD
6	Male	54	Squamous	Left upper lobe	Chemotherapy; radiotherapy	cT3N2M0 stage IIIB	CT	10^9 PFU + cisplatin	2	SD
7	Male	71	Squamous	Left upper lobe	Chemotherapy; radiotherapy	cT4N1M0 stage IIIB	Bronch	10^{10} PFU	2	PD
8	Male	52	Squamous	Right lower lobe	Chemotherapy; radiotherapy	cT2N2M0 stage IIB	Bronch	10^{10} PFU	1	Not assessable†
9	Male	66	Squamous	Left upper lobe	Surgery, laser; chemotherapy	cT2N0M0 stage IB; (pre-operative)	CT	10^{10} PFU	4	SD
10	Male	51	Adeno	Right lower lobe	Chemotherapy	cT2N2M1 stage IV	CT	10^{10} PFU + cisplatin	1	SD
11	Male	51	Adeno	Right upper lobe	Chemotherapy	cT4N3M0 stage IIIB	CT	10^{10} PFU + cisplatin	2	SD
12	Male	61	Squamous	Right upper lobe	Chemotherapy; radiotherapy	cT4N2M0 stage IIIB	Bronch	10^{10} PFU + cisplatin	2	SD
13	Male	62	Squamous	Left mainstem bronchus	Chemotherapy; radiotherapy	cT4N3M0 stage IIIB	Bronch	10^{11} PFU	1	Not assessable†
14	Male	70	Squamous	Left upper lobe	Chemotherapy	cT2N2M0 stage IIIA	Bronch	10^{11} PFU	4	SD
15	Male	62	Squamous	Right upper lobe	Chemotherapy; radiotherapy	cT4N2M0 stage IIIB	Bronch	10^{11} PFU	4	SD

Abbreviations: Squamous, squamous cell carcinoma; Bronch, bronchoscopic injection; PFU, plaque-forming units; PR, partial response; CT, computed tomography (CT)-guided injection; SD, stable disease; Laser, laser therapy; Adeno, adenocarcinoma.
*Introgen Therapeutics Inc, Houston, TX.
†Tumor sizes could not be measured due to obstructive pneumonia.

months). Two patients (13.3%) were not assessable because their tumor sizes could not be measured due to obstructive pneumonia. Of 13 assessable patients, three patients assessed as having partial response or SD received more than six cycles of ADVEXIN injection alone (patients 1 and 2) or in combination with cisplatin (patient 4) before disease progression. Symptomatic improvement, including reduction in dyspnea, cough, or hemoptysis was observed in four patients (26.7%) with refractory disease. One patient (patient 10) received additional external radiation therapy to 60 Gy over 6 weeks after two intratumoral injections of ADVEXIN and systemic administration of cisplatin, and survived for 3.9 months with evidence of the tumor in the right upper lobe. Overall survival analysis by Kaplan and Meier is 40% at 1 year, 13% at 2 years, and 7% at 3 years (Fig 1).

The first responding patient (patient 1) was a 58-year-old male with locally advanced squamous cell carcinoma at the tracheal bifurcation. He was unable to undergo surgery because of poor pulmonary function and received standard chemoradiotherapy. At the time of enrollment, he had relapsed endobronchial tumor in the area from the carina to the left mainstem bronchus, and received ADVEXIN at the 10^9 PFU dose level every 28 days. Four courses of bronchoscopic injection of ADVEXIN at the 10^9 PFU dose level every 4 weeks resulted in a marked tumor regression (Fig 2A) and relief of his symptoms such as hemoptysis, cough, and dyspnea. Pathologic examination demonstrated squamous metaplasia in most lesions, although there was a microscopic residual tumor in the left mainstem bronchus. He received a total of 14 courses of treatment, during which time no tumor progression was noted for approximately 11 months. His treatment was finally discontinued because of additonal local tumor progression.

The second responding patient (patient 2) presented with locally advanced unresectable squamous cell carcinoma that was completely obstructing the left lower lobe. After two courses of broncoscopic ADVEXIN injection at 10^9 PFU, radiographic tumor destruction was observed at the central portion (Fig 2B), resulting in reopening of the airway. He showed a significant improvement in breathing; treatment was, however, discontinued after nine courses of injections when distant bone metastasis was diagnosed.

A long-term SD was documented in a 46-year-old female with adenocarcinoma at the left upper lobe with multiple intrapulmonary metastasis (patient 4). She had disease progression during chemotherapy using cisplatin, vincristine, and etoposide as noted by a radiographic increase during the preceding 3 months, and received CT-guided injection of ADVEXIN at 10^9 PFU after systemic administration of cisplatin at 80 mg/m^2 every 28 days. Her primary adenocarcinoma in the left upper lobe as well as multiple pulmonary metastases were stable for approximately 10 months during the period of treatment (Fig 2C).

Immune Response to Adenovirus and p53

Fourteen of 15 patients (93.3%) were tested at baseline for neutralizing antibody to Ad5. Six of these patients showed low ($\leq$ 1:20) neutralizing antibody titers, and the other eight patients had titers ranging from 1:20 to 1:320. Titers of specimens collected at 4 weeks after the first injection of ADVEXIN were higher than the titers at baseline in 13 of 14 (92.9%) assessable patients, although the magnitude of increase in titer varied (Table 3). There was a correlation between the dose of the vector and the fold increase in the antibody

Table 2. Adverse Events Observed in Patients Who Received Injections of ADVEXIN*: Summary by Toxicity Grade

Toxicity	ADVEXIN Alone (n = 9) Grade 1	2	3	ADVEXIN + Cisplatin (n = 6) Grade 1	2	3	Total (N = 15) No.	%
Fever	1	7	1	0	5	0	14	93.3
Hemoptysis	6	1	0	2	0	0	9	60.0
Nausea	0	0	0	2	3	0	5	33.3
Vomiting	0	0	0	3	2	0	5	33.3
Cough	3	0	0	1	0	0	4	26.7
Diarrhea	2	0	0	2	0	0	4	26.7
Obstructive pneumonia	0	3	1	0	0	0	4	26.7
Constipation	2	0	0	1	0	0	3	20.0
Back pain	1	1	0	1	0	0	3	20.0
Chest pain	2	0	0	1	0	0	3	20.0
Anemia	0	1	0	0	0	2	3	20.0
Stomatitis	0	0	0	2	0	0	2	13.3
Shoulder pain	0	0	0	2	0	0	2	13.3
Infection	1	1	0	0	0	0	2	13.3
Fatigue	1	0	0	1	0	0	2	13.3
Lumbago	2	0	0	0	0	0	2	13.3
Paracusis	0	0	0	0	2	0	2	13.3
Dyspnea	0	0	1	0	0	0	1	6.7
Pneumonia	0	1	0	0	0	0	1	6.7
Atelectasis	0	0	1	0	0	0	1	6.7
Abdominal fullness	1	0	0	0	0	0	1	6.7
Dysesthesia	0	0	0	1	0	0	1	6.7
Pneumothorax	0	0	0	1	0	0	1	6.7
Hematoma	0	0	0	1	0	0	1	6.7
Pituita	0	0	0	1	0	0	1	6.7
Pleural effusion	1	0	0	0	0	0	1	6.7
Anal bleeding	1	0	0	0	0	0	1	6.7
Tooth pain	1	0	0	0	0	0	1	6.7
Bronchitis	0	0	0	0	1	0	1	6.7
Dysthymia	0	0	0	1	0	0	1	6.7
Injection site pain	0	0	0	1	0	0	1	6.7
Leukopenia	1	0	0	0	0	0	1	6.7
Anorexia	0	1	0	0	0	0	1	6.7
Weight loss	0	0	0	0	0	1	1	6.7
Finger abscess	0	0	0	1	0	0	1	6.7
Arm pain	0	0	0	0	1	0	1	6.7

NOTE. Toxicity defined by National Cancer Institute Common Toxicity Criteria (grade 1 to 4).
*Introgen Therapeutics Inc, Houston, TX.

titer (average/median fold increase in titer for 10^9; 10^{10}; 10^{11} PFU doses were: 27; 432; 516/16; 256; 516). Neutralizing antibody titers remained elevated in all patients assessed throughout the study. Two of 13 patients tested (patients 7 and 11) had detectable anti-p53 antibody levels at baseline. Anti-p53 titers increased after six courses and the first course of ADVEXIN administration in patients 2 and 4, respectively. The other nine patients remained negative for anti-p53 antibodies throughout the treatment.

Vector Shedding and Biodistribution

A total of 624 gargle and urine samples from 12 patients were examined for virus shedding by DNA-PCR using vector-specific primers (Table 4, Fig 2). Samples were collected just before vector injection and daily thereafter for 15 days in each course. Vector DNA

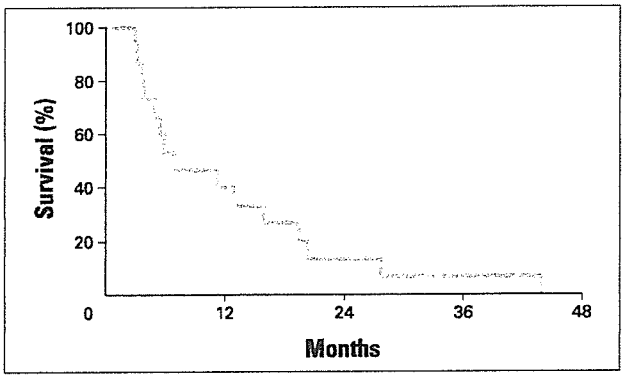

Fig 1. Kaplan-Meier analysis for overall survival of patients studied.

was detectable in 29 of 39 (74.4%) gargle samples obtained at day 1 after vector injection, regardless of dose level or treatment course, and declined to undetectable levels within 15 days for most patients. One patient (patient 11) was continuously positive for vector DNA until day 15 or beyond, probably due to the shedding of infected tumor cells. In total, 90 gargle samples (14.4%) were positive for vector DNA, whereas vector was detected in only 13 urine specimens (2.1%). The presence of ADVEXIN was assayed in the plasma obtained before and 30, 60, 90 minutes after vector injection (Fig 3). In all 12 patients tested, vector was detected in plasma 30 minutes after injection either by the DNA-PCR or CPE method, and the titers decreased over the next 60 minutes. No correlation was noted between systemic virus titer and AEs or clinical response.

Pathologic and Molecular Analysis of Tumor Biopsy Tissues

Tumor biopsy samples obtained before and 48 hours after vector injection in 12 patients were assessed for *p53* mRNA expression by RT-PCR analysis using vector-specific primers (Table 5). All pretreatment samples were negative for *p53* transgene expression because the primers can distinguish exogenous wt-*p53* from pre-existing mutant *p53* in tumor specimens. Vector-specific *p53* mRNA expression was detectable in 9 of 12 patients (75%) after the first vector injection. Overall, 11 of 12 patients (91.7%) demonstrated positive *p53* transgene expression during the treatment. Of note, *p53* mRNA expression was consistently positive in 52 of 57 serial postinjection samples (91.2%) obtained from patients who received more than six courses of injection (patients 1, 2, and 4). To evaluate the biologic activity of ADVEXIN-induced *p53* expression, we performed quantitative analysis of *p53*-targeted gene expression by a real-time RT-PCR method. Paired biopsy samples obtained before and 48 hours after injection at courses 1, 5, 8, and 11 were analyzed in patient 1 for expression of exogenous *p53*, *p21*, *Noxa*, and *p53AIP1* mRNAs. We found that apoptosis-related gene expression such as *Noxa* and *p53AIP1* was consistently higher in postinjection samples than that in preinjection biopsies throughout the study (Fig 4). The expression patterns of these mRNAs almost paralleled that of exogenous *p53*. In contrast, *p21* expression pattern varied on courses.

Autopsy results were obtained from two patients (patients 3 and 7) who died 25 and 151 days after receiving their fourth and second injection of the vector, respectively. DNA-PCR assay indicated that ADVEXIN was present in tumor tissue as well as proximal lymph nodes, suggesting regional spread of the vector via the lymphatic

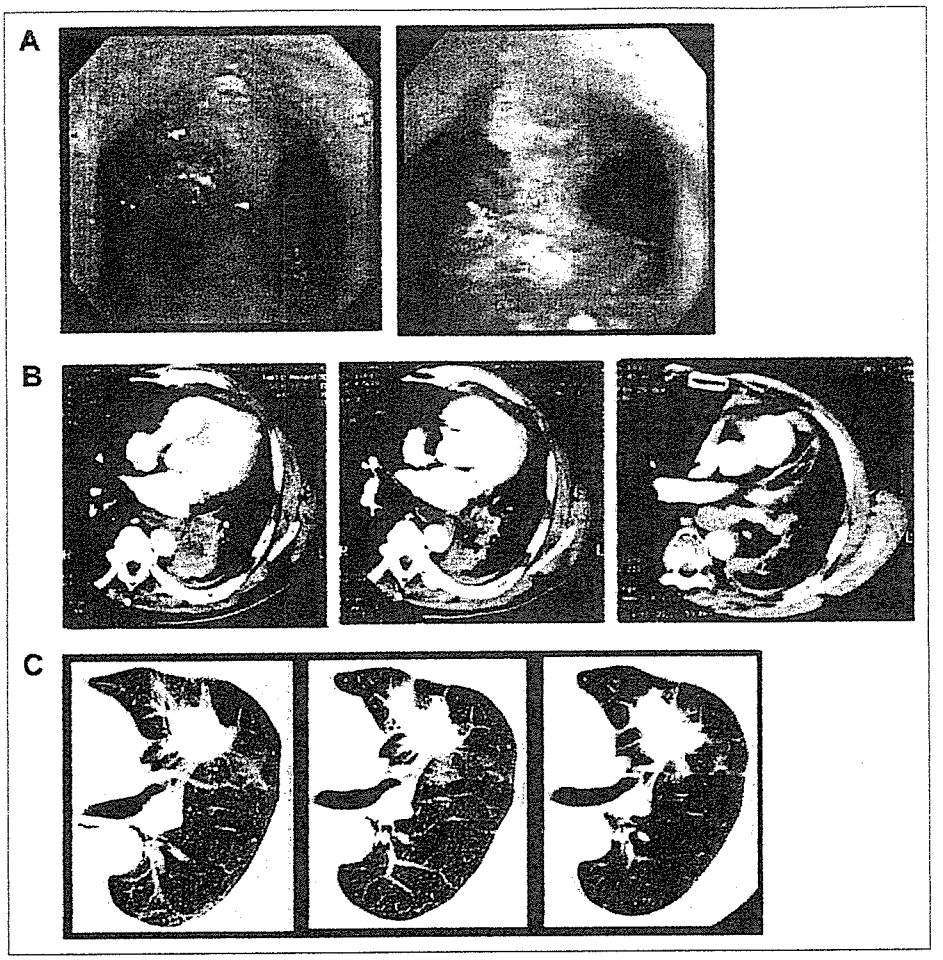

Fig 2. (A) Patient 1 bronchoscopic appearance at the time of enrollment (left) and after four cycles of injections (right). (B) Patient 2 computed tomography scans at baseline (left), at month 2 (middle), and at month 9 (right). (C) Patient 4 computed tomography scans at baseline (left), after four cycles (middle), and 10 cycles (right).

vessels. In contrast, viral distribution was not detected in other organs including liver, kidney, testis, and distal lymph nodes.

DISCUSSION

In an effort to determine the feasibility of *p53* gene therapy in human cancer treatments, several clinical trials of locoregional administration of adenoviral *p53* have been conducted in patients with a variety of advanced malignancies such as NSCLC,[18-20,22] head and neck cancer,[23] bladder cancer,[24,25] recurrent glioma,[26] and ovarian cancer.[27] No available data, however, has been reported yet evaluating this agent in Japanese patients. As clinical trials of the epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (Iressa; AstraZeneca, London, United Kingdom), revealed significant variability in the response to this drug, with higher responses seen in Japanese patients than in a predominantly European-derived population,[28] it is important to evaluate variations in the response and safety profiles of novel therapeutic agents in Japanese patients. The results of the present multicenter phase I trial indicate that intratumoral administration of ADVEXIN is feasible, safe, and well tolerated in Japanese patients.

Fever was observed as the most common treatment-related AE, which could be due to the transient systemic spread of the vector itself. In fact, flu-like symptoms, such as chills, fatigue or lethargy, and diarrhea, frequently occurred in patients who received replication-competent adenovirus[29,30]; these symptoms, however, are rarely seen in patients treated with ADVEXIN. Although the frequency of fever (affecting 93.3% of the patients) was slightly higher in our trial than those in the United States (79%) or European (76%) trials,[20,22] the observation that most episodes recovered within 48 hours after injection suggests that ADVEXIN-related AEs are limited, and only mild to moderate in severity.

The vector was shed into gargle specimens especially when bronchoscopically injected as expected, whereas vector was rarely detectable in urine despite of the systemic spread of the vector. Most PCR-positive gargle samples were negative by CPE assay (data not shown), indicating that secondary infection through vector shedding into gargle is unlikely to occur. The observation of systemic dissemination of ADVEXIN, which was maximal at 30 minutes after injection, is consistent with that observed in the United States trial, although the highest vector titer in the plasma was approximately $\log_1$ to $\log_2$ lower in Japanese patients.[21] The organ distribution of virus found in two deceased patients is clearly of interest, because, to the best of our knowledge, this is the first evidence of virus spread in the regional lymphatic tissues in humans. It has been reported that replication-deficient adenovirus expressing the *lacZ* gene could be

Table 3. Titers of Neutralizing Anti-Ad5 Antibody and Anti-p53 Antibody After ADVEXIN* Injections

Patient No.	Viral Dose ± Cisplatin	Antibody Titer or Positivity†						
		Baseline	Course 2	Course 3	Course 4	Course 5	Course 6	Course 7
Neutralizing anti-Ad5 antibody								
1	10^9 PFU	1:20	1:1,280	1:1,280	1:2,560	1:1,280	1:1,280	1:2,560
2	10^9 PFU	< 1:20	1:320	1:320	1:640	1:320	1:160	1:160
3	10^9 PFU	< 1:20	1:20	1:40	1:20	NA	NA	NA
4	10^9 PFU + cisplatin	1:320	1:160	1:1,280	1:1,280	1:1,280	1:1,280	1:5,120
5	10^9 PFU + cisplatin	1:40	> 1:20,480	1:2,560	NA	NA	NA	NA
6	10^9 PFU + cisplatin	< 1:20	1:2,560	NA	NA	NA	NA	NA
7	10^{10} PFU	1:20	1:20,480	NA	NA	NA	NA	NA
8	10^{10} PFU	1:20	1:5,120	NA	NA	NA	NA	NA
9	10^{10} PFU	1:320	1:5,120	1:10,240	1:10,240	NA	NA	NA
10	10^{10} PFU + cisplatin	1:40	> 1:20,480	NA	NA	NA	NA	NA
11	10^{10} PFU + cisplatin	< 1:20	1:80	1:5,120	NA	NA	NA	NA
12	10^{10} PFU + cisplatin	< 1:20	1:160	1:2,560	1:1,280	NA	NA	NA
13	10^{11} PFU	NA	NA	NA	NA	NA	NA	NA
14	10^{11} PFU	1:64	1:2,048	1:1,024	1:2,048	NA	NA	NA
15	10^{11} PFU	< 4	> 1:4,096	> 1:4,096	> 1:4,096	NA	NA	NA
Anti-p53 antibody								
1	10^9 PFU	–	–	–	–	–	–	–
2	10^9 PFU	–	–	–	–	–	–	+
3	10^9 PFU	–	–	–	–	NA	NA	NA
4	10^9 PFU + cisplatin	–	+	+	+	–	+	+
5	10^9 PFU + cisplatin	NA	NA	–	NA	NA	NA	NA
6	10^9 PFU + cisplatin	–	–	NA	NA	NA	NA	NA
7	10^{10} PFU	+	+	NA	NA	NA	NA	NA
8	10^{10} PFU	–	–	NA	NA	NA	NA	NA
9	10^{10} PFU	–	–	–	–	NA	NA	NA
10	10^{10} PFU + cisplatin	–	–	NA	NA	NA	NA	NA
11	10^{10} PFU + cisplatin	+	+	+	NA	NA	NA	NA
12	10^{10} PFU + cisplatin	–	–	–	–	NA	NA	NA
13	10^{11} PFU	NA	NA	NA	NA	NA	NA	NA
14	10^{11} PFU	–	–	–	–	NA	NA	NA
15	10^{11} PFU	NA	NA	NA	NA	NA	NA	NA

Abbreviations: PFU, plaque-forming units; NA, not available.
*Introgen Therapeutics Inc, Houston, TX.
†Samples were collected before each course of treatment.

transferred into regional lymph nodes of the stomach in dogs after intratumoral injection[31]; no lymph node tissues, however, were analyzed in any published clinical trials. This finding suggests that intratumorally administered ADVEXIN can spread not only into the blood circulation, but also into the lymphatic vessels, and potentially kill metastatic tumor cells in regional lymph nodes. Schuler et al[22] have reported that intratumoral adenoviral *p53* gene therapy provides no additional benefit in NSCLC patients receiving systemic chemotherapy by comparing the responses of injected lesions with those of noninjected comparator lesions, such as hilar metastatic tumors. According to our biodistribution data, the possibility that their virus spread into comparator lesions and showed antitumor effect cannot be ruled out.

Despite the presence of neutralizing antibodies for adenovirus, we found that *p53* transgene expression was detected in most patients throughout the period of treatment. In addition, quantitative real-time RT-PCR analysis demonstrated that the expression patterns of apoptosis-related *p53*-targeted genes such as *Noxa* and *p53AIP1* paralleled that of exogenous *p53* (Fig 4), demonstrating that exogenous *p53* produced after ADVEXIN treatment has biologic activity. These findings further demonstrate that circulating neutralizing anti-Ad5 antibodies do not inhibit vector-mediated transgene expression when ADVEXIN is directly injected into the tumors. Two of 13 patients (15.4%) had pre-existing anti-p53 antibodies, which is consistent with previous studies reporting that p53 autoantibodies were detected in 10% to 20% of patients with lung cancer.[32] Most patients, however, did not exhibit an increase of anti-p53 titers after ADVEXIN injection, presumably because of the short half-life of wt-p53 protein or due to lack of overcoming tolerance to this self protein.

As predicted by our preclinical and other clinical studies,[10-20] sustained antitumor effect was seen with tumor regression or stabilization of tumor growth in 11 of 13 assessable patients. Patient 1 exhibited a 50% or greater reduction in tumor size after ADVEXIN injection and this response was maintained with monthly injections; the growth rate of tumor, however, suddenly increased at 11 months after the time of entry, leading to the uncontrollable progressive disease. Although the molecular mechanism of this resistance to treatment is still under investigation, increasing the frequency of administration beyond once per month may be an approach to improve efficacy in such patients. Previous experience with ADVEXIN has shown that patients with airway stenosis or obstruction may be suitable candidates for this locoregional therapy.[18-20] Indeed, three patients (patients 1, 2, and 15) exhibited significant reopening of

Table 4. Vector Shedding Into Gargle and Urine After ADVEXIN® Injections

	DNA-PCR Results															
	Gargle (day)															
Course of Viral Dose ± Cisplatin	Pre	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10^9 PFU (patient 1)																
1	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−
2	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
4	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^9 PFU (patient 2)																
1	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
2	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−
3	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
4	−	+	−	−	+	−	−	+	−	−	−	−	+	−	−	−
5	−	+	+	+	−	−	−	−	−	−	+	−	−	−	−	−
6	−	+	−	−	+	−	+	−	−	+	−	+	−	−	−	−
10^9 PFU (patient 3)																
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^9 PFU + cisplatin (patient 4)																
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^9 PFU + cisplatin (patient 5)																
1	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
10^9 PFU + cisplatin (patient 6)																
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{10} PFU (patient 7)																
1	−	+	+	+	−	+	−	−	−	−	−	−	−	−	−	−
2	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{10} PFU (patient 8)																
1	−	+	−	−	−	+	−	+	+	−	−	−	−	+	−	−
10^{10} PFU (patient 9)																
1	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
4	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10^{10} PFU + cisplatin (patient 10)																
1	−	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−
10^{10} PFU + cisplatin (patient 11)																
1	−	+	−	+	−	−	−	−	+	−	−	−	+	−	−	−
2	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{10} PFU + cisplatin (patient 12)																
1	−	+	+	−	+	−	+	−	−	−	−	−	−	−	−	−
2	−	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−
10^9 PFU (patient 1)																
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

(continued on following page)

Table 4. Vector Shedding Into Gargle and Urine After ADVEXIN* Injections (continued)

Course of Viral Dose ± Cisplatin	DNA-PCR Results: Urine (day)															
	Pre	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
10^9 PFU (patient 2)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^9 PFU (patient 3)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^9 PFU + cisplatin (patient 4)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^9 PFU + cisplatin (patient 5)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^9 PFU + cisplatin (patient 6)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^{10} PFU (patient 7)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
2	–	–	–	–	–	–	–	–	–	+	–	+	+	+	+	–
10^{10} PFU (patient 8)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^{10} PFU (patient 9)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	+	+	+	+	+	–	–	+	–	–	–	–	–
3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^{10} PFU + cisplatin (patient 10)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^{10} PFU + cisplatin (patient 11)																
1	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10^{10} PFU + cisplatin (patient 12)																
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Abbreviations: PCR, polymerase chain reaction; PFU, plaque-forming units.
*Introgen Therapeutics Inc, Houston, TX.

the airway after bronchoscopic injection of ADVEXIN, and resulted in the marked improvement of symptoms such as cough, hemoptysis, and dyspnea.

It is of interest that three of six patients who were assigned to the cohort with the lowest dose of ADVEXIN could receive more than six courses of treatment, whereas no patients treated with higher doses completed six cycles of injections. There was a possible selection bias of patients related to the dose levels and the centers; this finding, however, suggests that 10^9 PFU of ADVEXIN appears to be sufficient to induce local effect without toxicities in certain patients. Another surprising observation from this study is that there was no apparent difference in clinical activity between the groups with ADVEXIN alone and ADVEXIN plus cisplatin. One possible explanation for this result is that cisplatin may affect induction of systemic immune response against mutant-p53–expressing tumor cells triggered by ADVEXIN. Support for this hypothesis is found in the reduced levels of anti-Ad5 antibodies in the high-dose ADVEXIN plus cisplatin group compared with high-dose

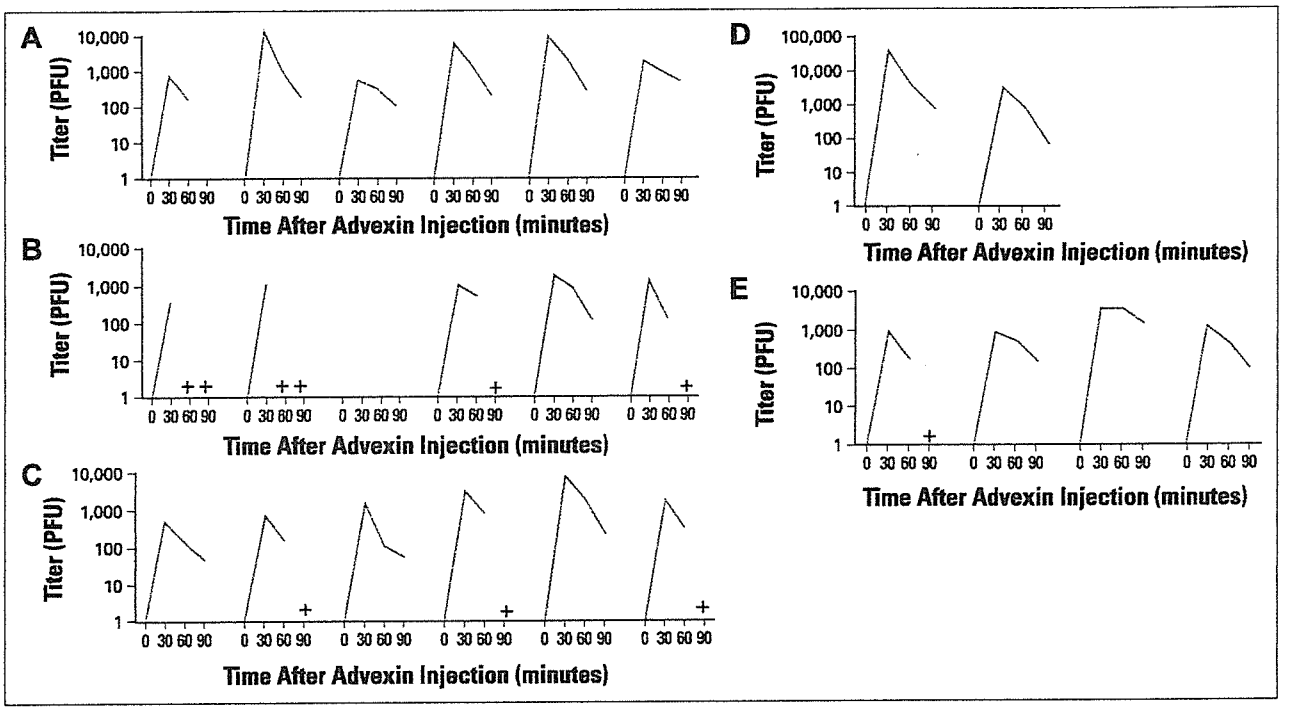

Fig 3. Quantitative detection of ADVEXIN (Introgen, Houston, TX) the cytopathic effect (CPE) assay in the plasma obtained before and 30, 60, and 90 minutes after vector injection. (A) Patient 1; (B) patient 2; (C) patient 4; (D) patient 7; and (E) patient 9. (+) Indicates negative by the CPE assay, but positive by a DNA-polymerase chain reaction analysis.

ADVEXIN as monotherapy. Furthermore, the study was not powered to identify differences in these groups.

In conclusion, this study demonstrates that repeated intratumoral injections of ADVEXIN in combination with or without cisplatin are feasible and well tolerated in Japanese patients. Despite undergoing more treatment cycles than other trials, patients experienced no severe toxicities and exhibited a long-term clinical activity ($\geq$ 9 months in 20% of patients). Therefore, it seems to be reasonable to explore the antitumor effect of this virus in patients with earlier-stage disease. This study provides support for the ongoing randomized phase III trials to identify the therapeutic benefit of ADVEXIN in squamous cell carcinoma of the head and neck and suggest that additional evaluations for NSCLC in phase II/III trials with appropriate controls are warranted.

Table 5. Vector-Specific *p53* mRNA Expression in Tumor Tissues After ADVEXIN* Injections

Patient No.	Viral Dose ± Cisplatin	RT-PCR Results Course 1	Course 2	Course 3	Course 4	Course 5	Course 6	Course 7	Course 8	Course 9	Course 10	Course 11	Course 12	Course 13	Course 14
1	10^9 PFU	−/+*	±	+/+	±	+/+	+/+	+/+	−/+	−/+	+/+	+/+	±	+/+	+/NA
2	10^9 PFU	−/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/NA					
3	10^9 PFU	−/−	−/−	+/+	+/+										
4	10^9 PFU + cisplatin	−/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/NA					
5	10^9 PFU + cisplatin	−/+	+/+	+/+											
6	10^9 PFU + cisplatin	−/−	−/−												
7	10^{10} PFU	−/−	−/−	+/NA											
8	10^{10} PFU	−/+	−/NA												
9	10^{10} PFU	−/+	−/+	±	+/+										
10	10^{10} PFU + cisplatin	−/+													
11	10^{10} PFU + cisplatin	−/+	+/+	+/ NA											
12	10^{10} PFU + cisplatin	−/+	+/+	−/+	−/NA										

NOTE. Paired samples were collected before and 48 hours after AVEXIN injection in each course of treatment.
Abbreviations: PFU, plaque-forming units; NA, not available.
*Introgen Therapeutics Inc, Houston, TX.

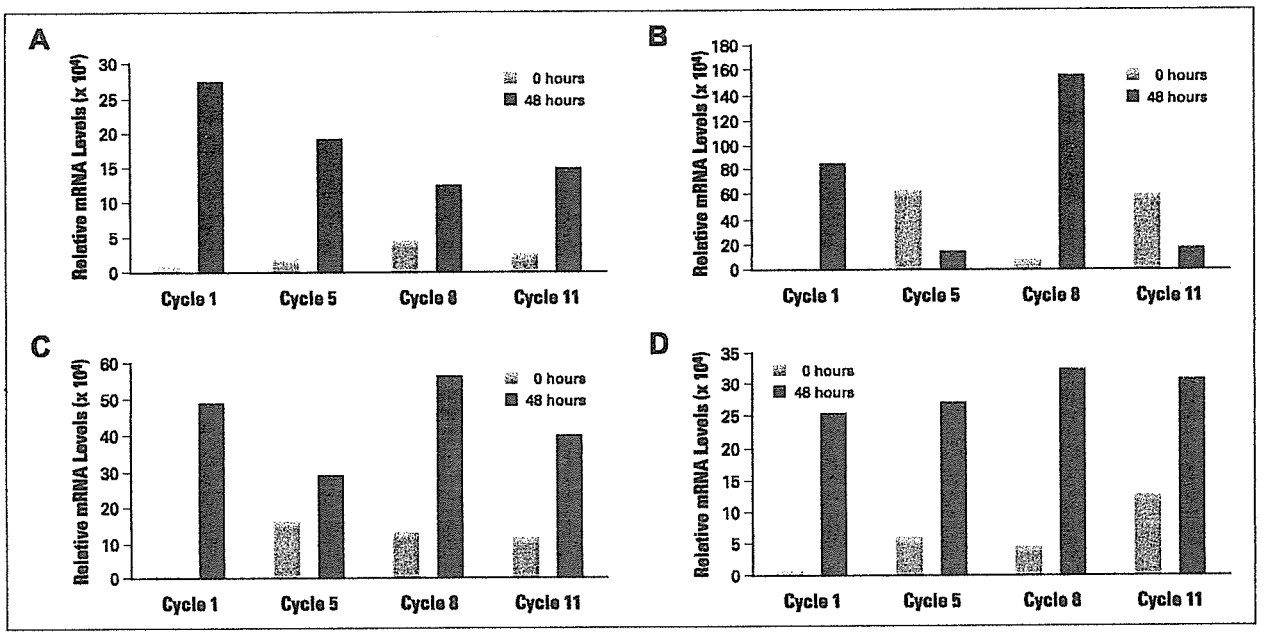

Fig 4. Expression of exogenous *p53* mRNA and *p53*-targeted gene mRNAs (*p21*, *Noxa*, and *p53AIP1*) was measured by the real-time quantitative polymerase chain reaction assay. (A) *p53*, (B) *p21*, (C) *Noxa*, and (D) *p53AIP1*. Tumor biopsy samples were obtained before and 48 hours after ADVEXIN (Introgen Therapeutics Inc, Houston, TX) injection at the indicated cycle (one, three, eight, and 11) in patient 1. All expression levels are normalized to that of β-Globin.

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Authors' Disclosures of Potential Conflicts of Interest

Although all authors completed the disclosure declaration, the following author or immediate family members indicated a financial interest. No conflict exists for drugs or devices used in a study if they are not being evaluated as part of the investigation. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Authors	Employment	Leadership	Consultant	Stock	Honoraria	Research Funds	Testimony	Other
Sunil Chada	Introgen Therapeutics (N/R)							
Dollar Amount Codes (A) < $10,000 (B) $10,000-99,999 (C) ≥ $100,000 (N/R) Not Required								

Author Contributions

Conception and design: Toshiyoshi Fujiwara, Noriaki Tanaka, Sunil Chada
Administrative support: Toshiyoshi Fujiwara, Yasuo Saijo, Toshihiro Nukiwa, Kunihiko Yoshimura, Tetsuo Sato, Yoshikatsu Eto, Haruhiko Nakamura, Harubumi Kato
Provision of study materials or patients: Toshiyoshi Fujiwara, Noriaki Tanaka, Susumu Kanazawa, Yasuo Saijo, Toshihiro Nukiwa, Kunihiko Yoshimura, Tetsuo Sato, Yoshikatsu Eto, Haruhiko Nakamura, Harubumi Kato
Collection and assembly of data: Toshiyoshi Fujiwara, Yasuo Saijo, Toshihiro Nukiwa, Kunihiko Yoshimura, Tetsuo Sato, Yoshikatsu Eto, Haruhiko Nakamura, Harubumi Kato
Data analysis and interpretation: Toshiyoshi Fujiwara, Shoichiro Ohtani, Sunil Chada
Manuscript writing: Toshiyoshi Fujiwara, Sunil Chada
Final approval of manuscript: Toshiyoshi Fujiwara, Noriaki Tanaka, Susumu Kanazawa, Yasuo Saijo, Toshihiro Nukiwa, Kunihiko Yoshimura, Tetsuo Sato, Yoshikatsu Eto, Sunil Chada, Haruhiko Nakamura, Harubumi Kato

GLOSSARY

Adenoviral p53: The E1- and E3-deleted, replication-deficient adenovirus vector encoding for the wild-type *p53* tumor suppressor gene.

Apoptosis: Also called programmed cell death, it is a signaling pathway that leads to cellular suicide in an organized manner. Several factors and receptors are specific to the apoptotic pathway. The net result is that cells shrink, develop blebs on their surface, and their DNA undergoes fragmentation.

Bystander effect: The biologically positive response observed in untreated cells when neighboring cells are treated. Because of the bystander effect, the magnitude of the therapeutic response exceeds the effect expected from direct target-cell treatment, illustrating that the treatment not only induces direct cytotoxic effects against the individual target cell but also causes the growth suppression of bystander, untreated cells via other mechanisms.

CPE (cytopathic effect): Viruses can infect target cells and cause cell death, referred to as the CPE. A CPE assay can be used to determine the titer of viral stocks.

Noxa: *Noxa* is a proapoptotic member of the *Bcl-2* family, which contains the *Bcl-2* homology 3 (BH3) region, but lacks other BH domains. *Noxa* functions as an early response gene and a mediator of p53-induced apoptosis. In human cells, Noxa is also designated as PMA-induced protein 1 or APR.

p21: The cyclin-dependent kinase inhibitor p21Cip1 inhibits cell-cycle progression by binding to cyclin/CDK complexes and arresting cells in the G1 phase of the cell cycle.

p53: The normal function of p53 is to act as a transcriptional activator of genes with a p53-binding site and an inhibitor of genes lacking a p53 binding site. Expression of high levels of wild-type p53 is associated with cell cycle arrest and apoptosis. Mutations in p53 are seen in several tumors.

p53AIP1 (p53-regulated, apoptosis-inducing protein 1): *p53AIP1* is a tumor suppressor gene that localizes to the mitochondria and regulates mitochondrial membrane potential.

RT-PCR (reverse-transcriptase polymerase chain reaction): PCR is a method that allows logarithmic amplification of short DNA sequences within a longer, double-stranded DNA molecule. Gene expression can be measured after extraction of total RNA and preparation of cDNA by a reverse-transcription step. Thus, RT-PCR enables the detection of PCR products on a real-time basis, making it a sensitive technique for quantitating changes in gene expression.

Tumor suppressor gene: A gene whose protein product is responsible for antiproliferative signals. The retinoblastoma gene product and the product of the p53 gene are two examples of tumor suppressor genes.

Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment

Yukari Muguruma, Takashi Yahata, Hiroko Miyatake, Tadayuki Sato, Tomoko Uno, Jobu Itoh, Shunichi Kato, Mamoru Ito, Tomomitsu Hotta, and Kiyoshi Ando

Hematopoiesis is maintained by specific interactions between both hematopoietic and nonhematopoietic cells. Whereas hematopoietic stem cells (HSCs) have been extensively studied both in vitro and in vivo, little is known about the in vivo characteristics of stem cells of the nonhematopoietic component, known as mesenchymal stem cells (MSCs). Here we have visualized and characterized human MSCs in vivo following intramedullary transplantation of enhanced green fluorescent protein-marked human MSCs (eGFP-MSCs) into the bone marrow (BM) of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Between 4 to 10 weeks after transplantation, eGFP-MSCs that engrafted in murine BM integrated into the hematopoietic microenvironment (HME) of the host mouse. They differentiated into pericytes, myofibroblasts, BM stromal cells, osteocytes in bone, bone-lining osteoblasts, and endothelial cells, which constituted the functional components of the BM HME. The presence of human MSCs in murine BM resulted in an increase in functionally and phenotypically primitive human hematopoietic cells. Human MSC-derived cells that reconstituted the HME appeared to contribute to the maintenance of human hematopoiesis by actively interacting with primitive human hematopoietic cells. (Blood. 2006; 107:1878-1887)

Introduction

Mesenchymal stem cells (MSCs) present in bone marrow (BM) are thought to give rise to cells that constitute the hematopoietic microenvironment (HME).[1] MSCs have been isolated from BM and various tissues from humans and many other species, expanded in culture, and shown to differentiate into osteocytes, chondrocytes, adipocytes, and myoblasts under defined conditions in vitro.[2] In culture, MSCs produce a number of cytokines and extracellular matrix proteins and express cell adhesion molecules, all of which are involved in the regulation of hematopoiesis.[3,4] They also support the development of hematopoietic colonies in vitro.[4] However, in contrast to hematopoietic stem cells (HSCs) that have been prospectively isolated and extensively studied at the single-cell level both in vitro and in vivo, MSCs have only been defined and isolated by physical and functional properties in vitro. Consequently, little is known about their phenotypic and functional characteristics in vivo.

Systemic administration of MSCs for facilitation of bone marrow transplantation has been proposed based on the in vitro characteristics of MSCs.[5] In recent studies, cotransplantation of human MSCs and HSCs resulted in increased chimerism or accelerated hematopoietic recovery (or both) in animal models and in humans,[6-9] suggesting a role for MSCs in the engraftment and repopulation of HSCs. Although the existence of donor MSCs has been documented in the BM of recipient animals following MSC infusion,[9,10] the methods used to detect engraftment, such as polymerase chain reaction (PCR) or staining of cytospin samples, could not unambiguously distinguish engraftment from cell survival or nonspecific lodgment on the vascular bed. In addition, Awaya et al examined stromal cells of patients who received BM transplants and confirmed that all donor signals were, in fact, derived from macrophages.[11] To our knowledge, there is no physical evidence that transplanted human MSCs have indeed engrafted in the BM of adult animals and directly participated in the enhanced engraftment of HSCs.

To assess the engraftment, spatial distribution, and lineage commitment of MSCs as well as their roles in hematopoiesis in vivo, we transplanted enhanced green fluorescent protein (eGFP)–marked human MSCs into the tibiae of nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice by intra–bone marrow transplantation (IBMT), a method previously shown to improve the engraftment of both hematopoietic and nonhematopoietic cells in mice.[12-14] We used a dual-color genetic marking strategy[15] along with immunofluorescent staining to distinguish and investigate transplanted cells in situ. We show that transplanted human MSCs integrated into the functional components of the HME and that these MSC-derived cells appeared to be actively involved in the maintenance of human hematopoiesis in murine BM.

From the Division of Hematopoiesis, Research Center of Regenerative Medicine, Department of Hematology, Teaching and Research Support Center, Department of Cell Transplantation and Regenerative Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan; and the Central Institute for Experimental Animals, Kawasaki, Kanagawa, Japan.

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Reprints: Kiyoshi Ando, Department of Hematology, Tokai University School of Medicine, Boseidai, Isehara, Kanagawa, 259-1193, Japan; e-mail: andok@keyaki.cc.u-tokai.ac.jp.

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Materials and methods

Isolation of human cord blood CD34+ cells

Human umbilical cord blood (CB) samples were obtained from full-term deliveries with informed consent of the mother and used in accordance with the institutional guidelines approved by the Tokai University Committee on Clinical Investigation. $CD34^+$ cells were selected using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Sunnyvale, CA) according to the manufacturer's instructions as described previously.[12] The purity of selected cord blood $CD34^+$ (CBCD34) cells was always greater than 95%, and they were cryopreserved in liquid nitrogen until use. In some experiments, CBCD34 cells were further fractionated into $CD34^+CD38^+$ and $CD34^+CD38^-$ populations at the day of transplantation.

Human MSCs

Human MSCs were purchased from Cambrex BioScience Walkersville (Walkersville, MD) and cultured according to the directions supplied by the company. The ability to differentiate into adipocytes, chondrocytes, and osteoblasts was confirmed in vitro before they were used for the experiments.[2]

Antibodies

The following antibodies were used for tissue immunostaining: anti-CD15 (80H5, 1:75; Coulter/Immunotech, Marseille, France), anti-CD31 (1:100; TECNE, Minneapolis, MN), anti-CD34 (My10, 1:20; BD Biosciences, San Jose, CA), anti-CD45 (2D1, 1:75; BD Biosciences), anti–glycophorin A (JC159, 1:400; Dako, Glostrup, Denmark), anti–N-cadherin (1:20; IBL, Gunma, Japan), antiosteocalcin (1:25; Biogenesis, Poole, United Kingdom), anti–smooth muscle (SM) actin (1A4, 1:800; Sigma-Aldrich, St Louis, MO), anti–alkaline phosphatase (B4-78, 1:30; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), antivimentin (1:400; Progen Biotechnik, Heidelberg, Germany), anti-GFP (1:500; MBL, Nagoya, Japan), antiosteopontin (10A16, 1:100; IBL), and anti–SDF-1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). The following monoclonal antibodies (mAbs) were used for flow cytometry: fluorescein isothiocyanate–conjugated anti-CD19 (SJ25C1; BD Biosciences), phycoerythrin-conjugated anti-CD33 (WM53) and anti-CD34 (581), and allophycocyanin-conjugated anti-CD45 (J.33; all from Coulter/Immunotech).

Experimental animals, lentiviral gene transduction, and cell transplantation

Eight- to 10-week old male NOD/Shi-*scid* (NOD/SCID) mice were purchased from Clea Japan (Tokyo, Japan) and housed in sterile microisolator cages in the animal facility of Tokai University School of Medicine. Mice were given autoclaved food and water. Twenty-four hours before transplantation, mice were irradiated with 300 cGy from an x-ray irradiator (HW-300, Hitex, Osaka, Japan) and thereafter fed acidified water. All procedures were approved by the Animal Care Committee of Tokai University. Prior to transplantation into mice, MSCs and CBCD34 were genetically marked with eGFP or its yellow variant enhanced yellow fluorescent protein (eYFP). Transduction of CBCD34 and MSCs was carried out as described previously.[15,16] For in situ examination of transplanted cells, 1×10^6 eGFP-marked MSCs and 2×10^5 eYFP-marked CBCD34, 2 to 4×10^5 $CD34^+CD38^+$ cells, or 1.5 to 4×10^4 $CD34^+CD38^-$ cells were suspended in 10 μL PBS and transplanted directly into the right tibia of NOD/SCID mice using a Hamilton syringe equipped with a 31-gauge needle.[12] In some experiments, gene-marked MSCs or CBCD34 cells were separately transplanted by IBMT or by the intravenous route.

Analysis of human cell homing

Staining of CBCD34 cells with PKH26 dye (Sigma-Aldrich) and analysis of cells that homed into BM were conducted as described previously.[12] Each mouse received 1×10^6 MSCs into the right tibia and 10 μL PBS into the left tibia by IBMT, followed by administration of 1×10^6 PKH26-labeled CBCD34 cells into the retro-orbital plexus. Twenty hours after transplantation, mice were humanely killed, and BM cells were collected separately from the tibiae that had been injected with MSC and the PBS.

Analysis of human cell engraftment

A total of 1×10^6 MSCs was injected into the right tibiae of irradiated NOD/SCID mice, and then 5×10^4 CBCD34 cells were injected into the retro-orbital plexus of the mice. Control groups received the same amount of PBS in the right tibia. At 6 weeks after transplantation, mice were humanely killed, and BM cells were collected separately from each tibia. Aliquots of cells were used to examine the percentages of CD45-, CD19-, CD33-, and CD34-expressing cells in the respective tibia. Two-color flow cytometric analysis was conducted using FACSCalibur. Quadrants were set to include at least 97% of the isotype-negative cells. The proportion of each lineage was calculated from 20 000 events acquired using CellQuest software (Becton Dickinson, San Jose, CA). Remaining cells were saved for the clonogenic cell assay and secondary transplantation.

Clonogenic cell assay

Human hematopoietic cells were isolated from BM cells of mice given transplants using CD45 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. The purity of selected cells was 36% to 96% (mean, 73%). CD45-enriched populations containing 10 000 $CD45^+$ cells were plated in MethoCult GFH4434V (StemCell Technologies, Vancouver, BC, Canada). The number and morphology of colonies formed during the 14-day culture period were determined under inverted microscope. Morphologic designation of colony type by light microscopy was confirmed by Wright-Giemsa staining of cytospin preparations. The specificity of the assay was confirmed by PCR on individual colonies using primers specific for the human chromosome 17-α satellite sequence[12] and the expression of CD45 by flow cytometric analysis.

Secondary transplantation

BM cells obtained separately from each tibia of primary mice were intravenously transplanted into irradiated secondary recipient mice. Because the number of cells recovered from one tibia was small (2.4-3.5 $\times 10^6$/tibia), we used NOD/SCID IL-2Rγ^{null} mice, which have been shown to be a better recipient of human cells than NOD/SCID mice,[17,18] as secondary recipients. Six weeks after transplantation, BM cells were obtained from tibiae and femurs of each secondary recipient, and the presence of human cells was analyzed by flow cytometry.

Tissue processing and immunofluorescent staining

Anesthetized mice were perfused with 4% paraformaldehyde in PBS. The tibiae were excised, decalcified, infiltrated with sucrose, embedded in OCT compound (Sakura, Tokyo, Japan), and frozen in liquid nitrogen. Frozen sections of decalcified bone were obtained using a cryostat microtome (CM3050, Leica, Germany) and stored at −80°C until staining. Immunofluorescent staining and enzyme immunohistochemistry were performed as described previously.[16] For immunofluorescent analysis, slides were examined and images were captured using an LSM510 META confocal microscope and a 63 ×/1.2 numeric aperture (NA) c-Apochromat objective lens (Carl Zeiss, Jena, Germany). Images were transferred to Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA).

Quantitative microscopic examination

Spatial distribution of human hematopoietic cells. The location of cells was designated as either endosteal (within 12 cells of the endosteum) or central (>12 cells of the endosteum) as described previously.[19] To accurately assess the proportion of endosteal area, the diameter of the diaphyseal shaft was evaluated by counting the number of cells from one side of the endosteum to the other along the line perpendicular to the longitudinal axis of the bone. CD34-, CD15-, and glycophorin A–reactive