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Specific Growth Inhibition of Small-cell Lung Cancer Cells by Adenovirus Vector Expressing Antisense c-*kit* Transcripts

Yuji Yamanishi, Hiroyuki Maeda, Keiko Hiyama, Shinichi Ishioka and Michio Yamakido

Second Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734

Antisense methods to control aberrant gene expression have been investigated as therapeutic strategies. A proto-oncogene c-*kit*, which encodes a transmembrane tyrosine kinase, is overexpressed in some malignancies, including small-cell lung cancer (SCLC), and is thought to be involved in their pathogenesis. To test the feasibility of using adenovirus vectors for antisense strategies and to target c-*kit* in SCLC therapy, we constructed replication-deficient recombinant adenovirus vectors which express fragments of c-*kit* transcripts in antisense (Ad.kitAS) or sense orientation (Ad.kitS: control). *In vitro* infection of SBC-1 cells, which are c-Kit protein-producing SCLC cells, by these vectors resulted in the expression of artificial c-*kit* transcripts. The Ad.kitAS-infected SBC-1 cells showed reductions in the amount of c-Kit protein. As expected, at 10 days after infection (1 multiplicity of infection), Ad.kitAS-infected SBC-1 cells showed approximately 40% growth inhibition compared to uninfected or Ad.kitS-infected cells *in vitro*. Such a significant growth inhibition by Ad.kitAS was not induced in SBC-5 cells, which are SCLC cells producing no c-Kit protein. These results demonstrate the usefulness of adenovirus vectors in antisense strategies, and the feasibility of targeting c-*kit* in the therapy of c-Kit-producing SCLC.

Key words: Adenovirus vector — Antisense strategy — c-*kit* — Small-cell lung cancer — Gene therapy

Lung cancer is one of the most common types of fatal malignancy, and its overall incidence is increasing.[1] Conventional treatments such as surgery, chemotherapy, radiotherapy, and immunotherapy are insufficiently effective, and the prognosis of lung cancer is very poor, with a 5-year survival rate of approximately 14%, and only 5% for small-cell lung cancer (SCLC).[1] Rapid advances in molecular oncology over the last decade have revealed that carcinogenesis results from multistage alterations of genes which play important roles in the regulation of cell proliferation and differentiation.[2–4] New therapeutic strategies to control the aberrant expression of dominant oncogenes[5–12] or tumor suppressor genes,[13–16] i.e., gene therapy, have therefore been studied vigorously with great expectations. To reduce overexpression of dominant oncogenes a number of methods have been developed, including intracellular antibodies,[5] dominant-negative mutations,[6] ribozymes,[7] and antisense strategies.[8–12]

The proto-oncogene c-*kit* encodes a transmembrane tyrosine kinase which is thought to play an important role in hematopoiesis, spermatogenesis, and melanogenesis.[17–19] Its expression is restricted to specific cells, such as $CD34^+$ human bone marrow cells, melanocytes, spermatogonia, and mammary epithelial cells in non-cancerous tissues.[20,21] On the other hand, c-*kit* expression has often been found in certain malignancies, such as hematopoietic malignancies,[22] SCLC,[23] and seminoma,[24] suggesting an important role in the pathogenesis of these malignancies. Furthermore, in SCLC cells, c-Kit is often co-expressed with its ligand, stem cell factor (SCF),[25] and is tyrosine-phosphorylated by binding with SCF, resulting in chemotaxis and cell proliferation.[26] Thus, the c-Kit-SCF system may form an autocrine or paracrine loop in the pathogenesis of SCLC. In this regard, c-*kit* may be a potential target of antisense therapeutics for SCLC.

With regard to antisense strategies, specific binding of antisense nucleotides to the target sequence results in the specific inhibition of gene expression via several mechanisms, including prevention of transcription,[27] processing,[28] and ribosomal translation[29] of the RNA, and target RNA degradation by provoking attacks by RNase.[30] Thus, cancer cell-specific treatment may be possible by targeting proto-oncogenes expressed exclusively or mainly in cancer.

Among a variety of methods which can be used to deliver antisense nucleotides to the target, adenovirus vector has an advantage by virtue of its high transduction efficiency *in vitro* and *in vivo*.[7,14–16,31–34] To evaluate the feasibility of an antisense strategy targeting c-*kit* using adenovirus vector for SCLC, we constructed a replication-deficient adenovirus vector which expresses antisense c-*kit* transcripts and investigated its inhibitory effect on the cell growth of SCLC *in vitro*.

MATERIALS AND METHODS

Cells The SCLC cell lines SBC-1 and SBC-5 were gifts from the Japanese Cancer Research Resources Bank. These cells were maintained in RPMI 1640 (Gibco BRL, Tokyo) containing 10% heat-inactivated fetal calf serum (FCS; Mitsubishi Kasei, Tokyo), 2 m*M* glutamine (Gibco BRL), 50 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified incubator in an atmosphere containing 5% CO_2.

Synthesis of human c-*kit* cDNA fragment Total cellular RNA was extracted from c-Kit protein-producing SBC-1 cells, using ISOGEN-LS (Nippon Gene, Tokyo) and subjected to reverse transcription (RT) using 6-bp random primers (You-Prime cDNA Synthesis Kit, Pharmacia, Uppsala, Sweden). The resulting cDNA was amplified by the polymerase chain reaction (PCR) method using the 5′-primer AGCTGATATCGATCCCATCGCAGCTACCGCGATG (1st to 24th nucleotides of Ref. 17) and the 3′-primer AGCTTCTAGAAACCTCAAGTCCTTGGGAAGAGGCTT (507th to 481st nucleotides of Ref. 17), so that the PCR products specifically correspond to a 507 bp sequence of human c-*kit* cDNA[17] including its translation initiation site, with an additional 19 bp of linker sequences. The sequence of synthesized c-*kit* cDNA fragment was confirmed by sequence analysis using a dsDNA Cycling Sequence kit (Gibco BRL).

Construction of recombinant adenovirus vectors The construction of replication-deficient recombinant adenovirus vectors was performed as described previously[33,34] (Fig. 1). First, the cytomegalovirus immediate early (CMV IE) promoter/enhancer, the synthesized human c-*kit* cDNA fragment in antisense (pCMVkitAS) or in sense (pCMVkitS) orientation, and the SV40 RNA maturation signals (SV40 small T intron and SV40 polyadenylation signals, both from pSV2CAT[35]) were inserted into the plasmid pXCJL.1 (a gift from Dr. F. Graham, McMaster University, Canada, Ref. 34). The resulting plasmids, pCMVkitAS or pCMVkitS, were then cotransfected with the plasmid pJM17[36] containing the adenovirus type 5 whole genome, into 293 cells, a transformed human embryonic kidney cell line (American Type Tissue Collection CRL 1573, Rockville, MD), by the calcium phosphate precipitation method.[37] As a consequence of homologous recombination between pCMVkitAS or pCMVkitS and pJM17, replication-deficient recombinant adenovirus vectors, containing CMV IE promoter/enhancer to drive c-*kit* cDNA fragments in the antisense (Ad.kitAS) or sense (Ad.kitS, a control vector) orientation, were generated. These adenovirus vectors were propagated in 293 cells and purified by cesium chloride gradient ultracentrifugation.[37] The viral titers were determined by both plaque-formation assay[37] and optical absorbance measurement at 260 nm.[31]

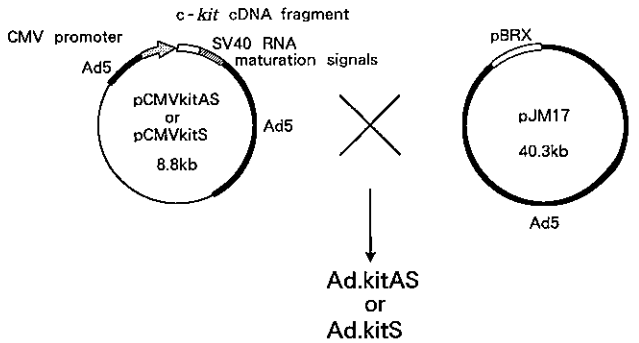

Fig. 1. Construction of recombinant adenovirus vectors. The plasmid pCMVkitAS or pCMVkitS were generated from the plasmid pXCJL.1 containing CMV IE promoter/enhancer, the human c-*kit* cDNA fragment (in antisense or sense orientation), and the SV40 RNA maturation signals. pCMVkitAS or pCMVkitS were cotransfected with the plasmid pJM17 into 293 cells to generate the recombinant adenovirus vectors Ad.kitAS or Ad.kitS.

In a similar manner, we constructed Ad.*lac*Z, containing *Escherichia coli lac*Z reporter gene instead of c-*kit* cDNA fragment in Ad.kitAS, to evaluate adenovirus-mediated gene transduction efficiency into SCLC cells.

***In vitro* infection with adenovirus vectors** When the cells had grown to 70–80% confluence, they were infected with adenovirus vectors at various values of multiplicity of infection (MOI) and the cells were incubated in infection media (RPMI 1640 containing 2 m*M* glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin) for 90 min at 37°C. The medium was then replaced with complete RPMI 1640 culture medium containing 10% FCS, and the cells were further incubated as specified. Uninfected cells were incubated simultaneously in a similar manner.

Adenovirus-mediated gene transduction efficiency in SCLC cells After 72 h incubation, at approximately 5×10^5 cells/ml, cells infected with Ad.*lac*Z at 20 MOI and uninfected cells were washed with phosphate-buffered saline (PBS; Nissui, Tokyo) and fixed with cold fixing solution (2% paraformaldehyde [Sigma, St. Louis, MO] and 0.2% glutaraldehyde [Sigma] in PBS at pH 7.4). The cells were then washed with PBS three times and placed in a solution containing 5 m*M* $K_4Fe(CN)_6$, 5 m*M* $K_3Fe(CN)_6$, 1 m*M* $MgCl_2$, and 1 mg/ml X-gal (Boehringer Mannheim, Indianapolis, IN) in PBS for 16 h.

Western blot analysis of c-Kit protein Approximately 10^7 cells of SBC-1 or SBC-5 were homogenized in 100 μl of lysis buffer (20 m*M* Tris-HCl pH 8.0, 137 m*M* NaCl, 10% glycerol, 1% Nonidet P-40, 1 m*M* phenylmethylsulfonyl fluoride [Sigma], 0.15 U/ml aprotinin [Sigma], 10 m*M* EDTA, and 10 μg/ml leupeptin [Sigma]) and incubated at 4°C for 20 min, then centrifuged at 10,000*g*

for 15 min at 4°C to remove debris. The protein concentrations in the cell lysates were determined by use of BCA protein assay reagent (Pierce, Rockford, IL), and then the lysates were mixed with sample buffer containing 2-mercaptoethanol and boiled for 5 min. Samples containing 30 μg total protein were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–20% polyacrylamide gradient gel (Multigel, Daiichi Pure Chemicals, Tokyo), and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon, Daiichi Pure Chemicals) by semi-dry electroblotting. The membrane was blocked for 2 h at 37°C in PBS with 0.05% Tween 20 (PBST), containing 5% fat-free dried milk. Rabbit anti c-Kit polyclonal antibody K963 (IBL, Fujioka) was added at a concentration of 10 μg/ml and incubated for 2 h at 37°C. Following 3 washes in PBST, avidin-biotin-peroxidase complex staining (Vectastain *Elite* ABC kit, Vector Laboratories, Burlingame, CA) was performed according to the manufacturer's instructions.

Northern blot analysis of artificial c-*kit* transcripts To demonstrate the artificial gene expression mediated by adenovirus vectors, SBC-1 cells were infected with Ad.kitS or Ad.kitAS at 1, 5, and 10 MOI. After 72-h incubation, total RNA was extracted from the cells. Ten micrograms of the RNA was electrophoresed on 1.5% agarose gels and transferred onto nitrocellulose filters. The filters were hybridized with a ^{32}P-labeled c-*kit* cDNA probe, which had been synthesized for the construction of Ad.kitAS, then washed with 0.1× standard saline-citrate-0.1% sodium dodecyl sulfate at 50°C, and autoradiographed. The same filters were then hybridized with a β-actin probe (Oncor, Gaithersburg, MD) to confirm that equal amounts of mRNA had been loaded in each lane.

Effect of antisense c-*kit* transcripts on c-Kit protein production To investigate the inhibitory effect of Ad.kitAS on c-Kit protein production, SBC-1 cells infected with Ad.kitS or Ad.kitAS at 1 and 5 MOI were prepared. After 6-day incubation, 10^7 cells infected with Ad.kitS or Ad.kitAS and uninfected cells were homogenized and subjected to Western blotting. The intensity of each 145 kD band, corresponding to c-Kit protein, was estimated by using a charge-coupled device (CCD) imaging system (Densitograph AE-6900 MF, ATTO, Tokyo).[38]

Cell growth curve Uninfected cells and those infected with Ad.kitS or Ad.kitAS were seeded in 6-well culture plates (Falcon 3046, Becton Dickinson, NJ) at 1.0×10^5 cells/2 ml/well, and cell numbers were counted using a hemacytometer at 1, 3, 5, 7, and 10 days. Cell viability was also judged with the trypan blue dye exclusion test. All experiments were done in triplicate.

Statistical analysis Data were analyzed using a statistical software package Statview II (Abacus Concepts, Berkeley, CA). Data were expressed as means±SD and means were compared by ANOVA. The criterion of statistical significance was $P<0.05$.

RESULTS

Adenovirus-mediated gene transduction efficiency in SCLC cells All of the SBC-1 and SBC-5 cells infected with Ad.*lac*Z at 20 MOI demonstrated *lac*Z expression without apparent cytopathic effect (CPE). No internal *lac*Z expression was observed in uninfected cells.

Production of c-Kit protein in SCLC cells Bands at approximately 145 kD, corresponding to c-Kit protein, were demonstrated only for SBC-1 cells, and not for SBC-5 cells (Fig. 2).

Expression of artificial c-*kit* transcripts mediated by adenovirus vectors Using Northern blot analysis, bands at approximately 1.45 kb, corresponding to artificial c-*kit* transcripts, were detected for SBC-1 cells infected with Ad.kitS or Ad.kitAS in a viral dose-dependent fashion (Fig. 3).

Reduction of c-Kit protein production by antisense c-*kit* transcripts Compared with uninfected cells, SBC-1 cells infected with Ad.kitAS showed 75% (1 MOI) and 92% (5 MOI) reduction of c-Kit protein on day 6 of incubation. At the same MOI, weaker reduction of c-Kit protein was observed in cells infected with Ad.kitS (25% at 1 MOI and 73% at 5 MOI, respectively) (Fig. 4, Table I). The reproducibility of this result was confirmed in triplicate experiments (data not shown).

***In vitro* cell growth inhibition** Aliquots of 1.0×10^5 uninfected SBC-1 cells and SBC-1 cells infected with

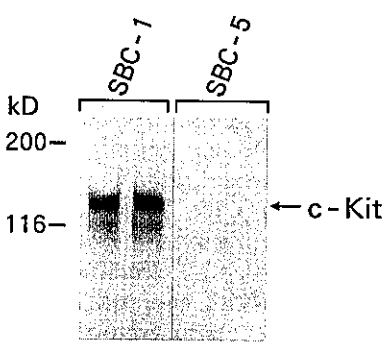

Fig. 2. Western blot analysis of c-Kit protein. Protein extracts were prepared from SBC-1 and SBC-5 cells. A total of 30 μg protein from each sample was subjected to SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with the polyclonal anti-c-Kit antibody. c-Kit protein expression (145 kD bands; arrow) was detected in SBC-1 cells, but not in SBC-5 cells (all experiments were done in duplicate).

Ad.kitS or Ad.kitAS at 1 MOI were incubated for up to 10 days after infection (Fig. 5A). SBC-1 cells infected with Ad.kitAS exhibited suppression of growth compared with those infected with Ad.kitS (36%, $P<0.05$), and uninfected cells (40%, $P<0.01$) on day 10 of incubation (Table II). In contrast to the effect on cell numbers, on day 7 of incubation the viability of SBC-1 cells infected with Ad.kitAS (64.2%) was higher than that of those which were infected with Ad.kitS (40.4%, $P<0.01$) or uninfected (43.3%, $P<0.01$) (Table II). On day 10 of incubation, when significant cell growth inhibition was observed in cells infected with Ad.kitAS, the viability of SBC-1 cells infected with Ad.kitAS (49.9%) was also higher than that of those which were infected with Ad.kitS (37.6%, not significant) or uninfected (38.7%, not significant) (Table II).

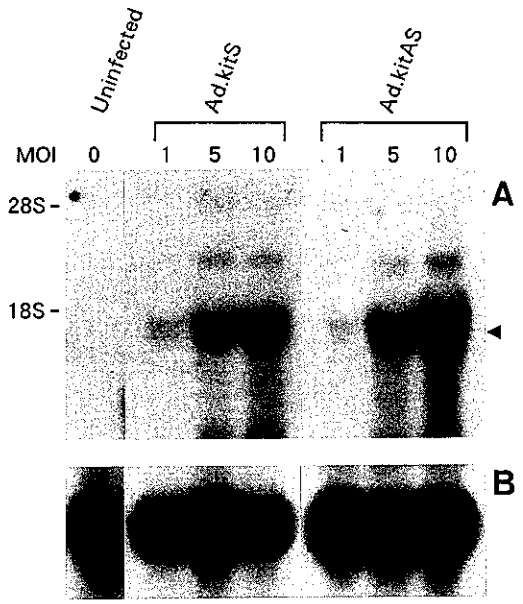

Fig. 3. Expression of artificial c-*kit* transcripts mediated by adenovirus vectors. Total RNA extracted from uninfected SBC-1 cells and SBC-1 cells infected with Ad.kitS or Ad.kitAS at 1, 5, and 10 MOI after 72 h incubation was prepared for Northern blot analysis. Ten micrograms of total RNA of each cell was electrophoresed on 1.5% agarose gels and then transferred onto nitrocellulose filters. The filters were hybridized with a ^{32}P-labeled c-*kit* cDNA probe, which had been synthesized for the construction of Ad.kitAS. Artificial c-*kit* transcripts (1.45 kb bands; arrowhead) were detected for SBC-1 cells infected with Ad.kitS or Ad.kitAS in a viral dose-dependent fashion (A). The same filters were then rehybridized with a β-actin probe to confirm equality of the amount of mRNA loaded in each lane (B).

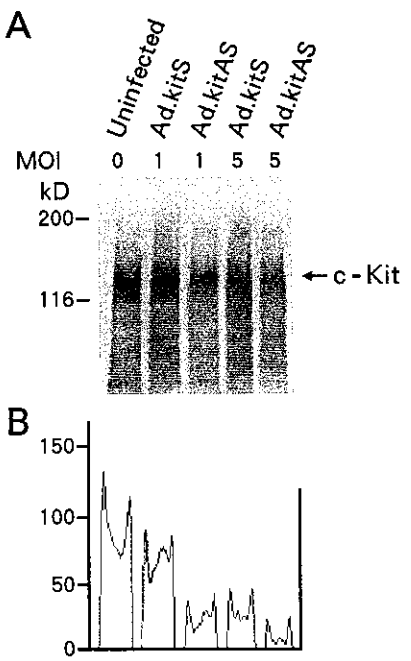

Fig. 4. Effect of Ad.kitAS on c-Kit protein production. Protein extracts were prepared from uninfected SBC-1 cells and SBC-1 cells infected with Ad.kitS or Ad.kitAS at 1 and 5 MOI on day 6 of incubation. A total of 90 μg protein from each sample was subjected to SDS-PAGE and transferred onto a PVDF membrane. The membrane was probed with polyclonal anti-c-Kit antibody. c-Kit protein expression (145 kD bands; arrow) was detected in all samples (A). The intensity of each 145 kD band was estimated using a CCD imaging system (B).

Table I. Effect of Ad.kitS and Ad.kitAS on c-Kit Protein Production in SBC-1 Cells

Cells	Uninfected	MOI=1		MOI=5	
		Ad.kitS	Ad.kitAS	Ad.kitS	Ad.kitAS
SBC-1[a)]	1987	1500	500	534	167
% reduction[b)]	—	25	75	73	92

a) Peak area of c-Kit protein band measured by the CCD imaging system using cells on day 6 of incubation.
b) Percentage reduction in c-Kit protein production compared to uninfected cells.

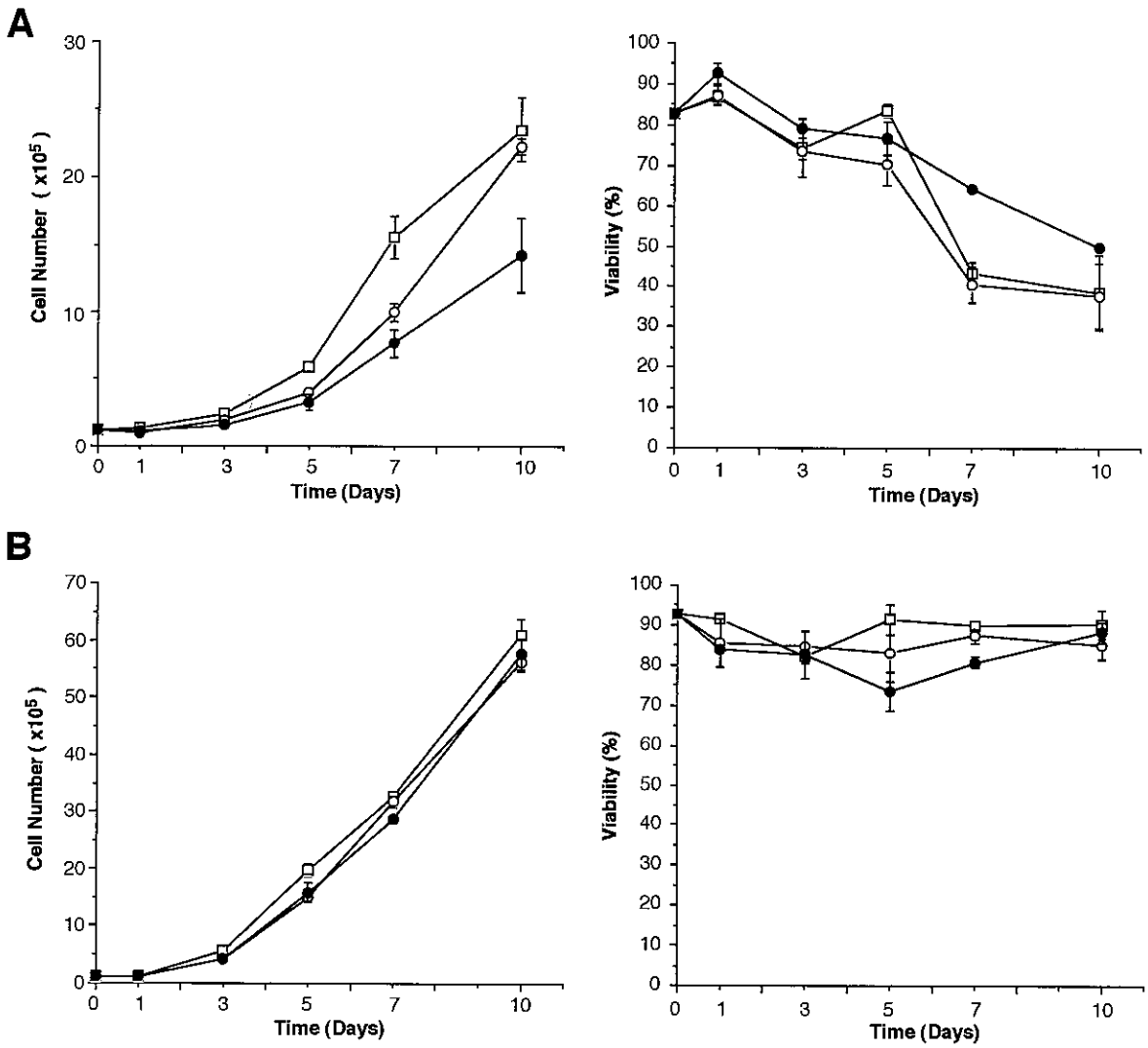

Fig. 5. Effects of Ad.kitAS on cell growth and viability of SBC-1 cells (A) and SBC-5 cells (B). Left: growth curves of cells infected with Ad.kitAS or Ad.kitS at 1 MOI, and of uninfected cells. Cells (1.0×10^5) were seeded in 6-well culture plates and cell numbers were counted periodically for 10 days using a hemacytometer. Right: cell viability curves. □, uninfected cells; ○, cells infected with Ad.kitS; ●, cells infected with Ad.kitAS. Data are displayed as means±SD (n= 3).

In a similar manner, 1.0×10^5 uninfected SBC-5 cells, which had been confirmed not to be producing c-Kit protein by Western blot analysis (Fig. 2), and SBC-5 cells infected with Ad.kitS or Ad.kitAS at 1 MOI were cultured for up to 10 days (Fig. 5B). There was no significant difference among them in the numbers of cells (Table II).

DISCUSSION

The present experiments demonstrate that recombinant adenovirus vectors have the potential to serve as efficient antisense gene delivery vehicles for antisense gene therapy, and that the expression of antisense c-*kit* gene specifically inhibits the cell growth of c-Kit protein-producing SCLC cells.

To utilize antisense strategies in the treatment of cancer, methods must be developed to mediate the highly efficient transduction of antisense nucleotides into cancer cells *in vivo*. Recently, adenovirus vector has been extensively studied as a delivery vehicle by virtue of its broad host spectrum and highly efficient gene transfer ability, both *in vitro* and *in vivo*.[7,14-16,31-34] Additionally, it has a very low efficiency of integration and is considered

Table II. Effects of Ad.kitS and Ad.kitAS on Growth and Viability of SCLC Cells

Cells	Uninfected	Ad.kitS	Ad.kitAS
SBC-1			
Cell number ($\times 10^5$)[a]	23.5±2.35	22.3±0.60	14.2±2.81[b, c]
Viability (%)			
day 7	43.3±3.01	40.4±4.41	64.2±0.85[b, c]
day 10	38.7±9.00	37.6±8.30	49.9±0.47
SBC-5			
Cell number ($\times 10^5$)[a]	61.0±2.76	56.1±1.63	57.5±2.61
Viability (%)			
day 7	90.1±0.15	87.4±1.95	80.1±1.41[b, c]
day 10	90.3±3.70	85.1±3.45	88.2±2.63

All data are means±SD of triplicate determinations.
a) Cell number on day 10 of incubation.
b) $P<0.05$ (ANOVA) compared to uninfected.
c) $P<0.05$ (ANOVA) compared to Ad.kitS.

not to be associated with insertional mutagenesis. However, no successful experiments on the application of adenovirus vector to antisense strategy have been reported. In this context, we investigated a new antisense strategy using recombinant adenovirus vector. Our data demonstrated that recombinant adenovirus vector, encoding antisense c-*kit* gene under the control of CMV IE promoter/enhancer, was capable of highly efficient transfer and expression of antisense c-*kit* gene in SCLC cells (Fig. 3).

In order to minimize the damage to normal host tissues, the use of therapeutic reagents with cancer cell-specific toxicity is desirable for human cancer gene therapy. For patients with SCLC, expression of c-Kit protein is thought to be restricted to SCLC tumors in the lungs, as no expression was found in normal lung or bronchus.[21] Therefore, by targeting the c-*kit* gene using the antisense approach by intratracheal administration to the lung, SCLC cell-specific treatment should be possible. Our *in vitro* results showed that Ad.kitAS selectively inhibited the c-Kit protein-producing SCLC cell growth of SBC-1, in which expression of SCF had been detected using the RT-PCR technique (data not shown), with little influence on SCLC cell growth of SBC-5 producing no c-Kit protein (Table II). In this experiment, since significant inhibition of cell growth by Ad.kitAS was observed only in c-Kit protein-producing cells, and since it was roughly proportional to the reduction in the amount of c-Kit protein, the growth inhibition was considered to arise from the reduction in c-Kit protein synthesis. The growth inhibitions of SBC-1 cells by Ad.kitAS were rather mild, probably due to the small amount of adenovirus vector (1 MOI). Although stronger growth inhibition could be expected by using more Ad.kitAS (5 MOI), we employed 1 MOI in order to minimize cytotoxicity by adenoviral infection. Cells seeded at low density, as in our experiments, are susceptible to environmental factors, including viral infection. In fact, SBC-1 cells infected with adenovirus vectors which were seeded at low density (0.5×10^5 cells/ml) showed CPE at 5, 25, and 50 MOI in our preliminary experiment (data not shown). On the other hand, after seeding at 5×10^5 cells/ml, no apparent CPE was observed in the cells infected with Ad.*lac*Z at 20 MOI. Furthermore, there have been numerous reports in which higher MOI was employed at higher cell density without significant problems.[14-16, 33] Further investigations are needed to determine the optimum dose of adenovirus vector for *in vivo* administration.

Throughout the period of incubation, the viability of antisense transfectants was not significantly lower than that of sense transfectants of SBC-1 cells. On the contrary, the viability of antisense transfectants was higher than that of uninfected cells and sense transfectants on day 7 ($P<0.01$) and day 10 (not significant) (Fig. 5). These findings suggested that the growth inhibition by Ad.kitAS was not due to non-specific viral effects, including CPE. Clusters formed by antisense transfectants were smaller in size than those formed by sense transfectants. In general, rapidly proliferating cells tend to form larger clusters than slowly growing cells, and as the clusters become larger, so the viability of the cells at the center of the clusters becomes lower, due to micro-environmental factors such as lack of nutrition. This could explain the fact that the viability of antisense transfectants was higher than that of controls in SBC-1 cells. In assays of growth inhibition of cluster-forming cells, it is thus important to evaluate both total cell numbers and viability.

Weaker suppression of c-Kit protein synthesis in sense transfectants was observed (Table I), and was thought to be due to virus infection. Adenovirus infection is known to suppress host cell protein synthesis via several

mechanisms such as inhibition of the translocation of newly synthesized and processed cellular mRNAs into the cytoplasm, and blockade of translation of host cell mRNAs.[39, 40] The amount of c-Kit protein in host cells should be readily and non-specifically reduced by adenovirus infection, because of its rapid turnover.[41] Such non-specific reduction of the protein synthesis due to viral infection may lead to non-specific inhibition of cell growth, as well as potentially increasing apoptotic cell death and lowering recruitment into S phase.[42] In this context, the precise quantification of the amounts of virus was critical to exclude non-specific effects of virus infection. Therefore, the titer of the virus preparation was carefully determined by two different methods in our study. Furthermore, equality in the amounts of adenovirus vector was demonstrated by the following facts. First, bands of artificial c-*kit* transcripts in cells infected at the same MOI were observed at equivalent intensity in Northern blot analysis (Fig. 3). Second, the viability of sense and antisense transfectants was almost equal on day 3 of incubation (Fig. 5). Our preliminary experiments (data not shown) and others[42] had revealed that non-specific reduction in viability was observed from 2 or 3 days after adenoviral infection, being proportional to the administered dose of adenovirus vectors.

One limitation of using adenovirus vectors is that since the genes transferred exist as extrachromosomal DNA, the amount of transgenes in the target cells decreases as the cells divide. However, this does not seem to hamper the capability of adenovirus vectors for treating cancer.[7, 14–16] In this experiment, adenovirus-mediated gene transfer of antisense c-*kit* inhibited cell growth at only 1 MOI, and the effect continued for at least 10 days. Kass-Eisler reported that 1 MOI is equal to approximately 100 adenoviral particles per cell, and that 1 MOI is sufficient for infection of all cardiac myocytes *in vitro*.[32] Accordingly, we considered that almost all SCLC cells were transfected by adenovirus vectors under the same condition. These findings explain the prolonged effect of the adenovirus vector used at such a low MOI on proliferating cancer cells in our experiment.

In conclusion, antisense c-*kit* RNA expressing adenovirus vector can specifically inhibit cell growth of c-Kit protein-producing SCLC cells by inhibition of c-Kit protein synthesis *in vitro*. It should be worthwhile to investigate further this strategy of gene therapy using antisense-gene-expressing recombinant adenovirus vectors with *in vivo* direct administration.

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