Adenovirus-mediated suppression of HMGI(Y) protein synthesis as potential therapy of human malignant neoplasias

Stefania Scala*, Giuseppe Portella*, Monica Fedele*, Gennaro Chiappetta†, and Alfredo Fusco‡§

*Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche c/o Dipartimento di Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università degli Studi di Napoli, 80131 Naples, Italy; †Istituto Nazionale dei Tumori Fondazione Senatore Pascale, via M. Semmola, 80131 Naples, Italy; and ‡Dipartimento di Medicina Sperimentale e Clinica, Facoltà di Medicina e Chirurgia di Catanzaro, Università degli Studi di Catanzaro, via Tommaso Campanella 5, 88100 Catanzaro, Italy

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High mobility group I (HMGI) proteins are overexpressed in several human malignant tumors. We previously demonstrated that inhibition of HMGI synthesis prevents thyroid cell transformation. Here, we report that an adenovirus carrying the HMGI(Y) gene in an antisense orientation (Ad-Yas) induced programmed cell death of two human thyroid anaplastic carcinoma cell lines (ARO and FB-1), but not normal thyroid cells. The Ad-Yas virus led to death of lung, colon, and breast carcinoma cells. A control adenovirus carrying the *lacZ* gene did not inhibit the growth of either normal or neoplastic cells. Ad-Yas treatment of tumors induced in athymic mice by ARO cells caused a drastic reduction in tumor size. Therefore, suppression of HMGI(Y) protein synthesis by an HMGI(Y) antisense adenoviral vector may be a useful treatment strategy in a variety of human malignant neoplasias, in which HMGI(Y) gene overexpression is a general event.

carcinomas | thyroid | antisense

Proteins HMG-I, HMG-Y, and HMGI-C constitute the high-mobility group I protein family. The first two proteins are encoded by the same gene, i.e., HMGI(Y), through alternative splicing (1, 2), whereas HMGI-C is the product of a different gene (3). They are nonhistone nuclear proteins, which bind AT-rich regions in the minor groove of DNA via three AT-hook domains. They are thought to affect transcription by acting as architectural proteins (4–6).

Overexpression of HMGI proteins is correlated with the appearance of a highly malignant phenotype in rat thyroid differentiated cells and in human and experimental thyroid tumors (7–11). Other human neoplasias (e.g., colon, prostate, and cervix carcinomas) also express high HMGI(Y) levels (12–15). Conversely, HMGI gene expression is negligible in normal adult tissues and is essentially restricted to embryonic development (2, 3, 15, 16). Transfection of normal rat thyroid cells with a vector carrying the HMGI-C gene in an antisense orientation resulted in the suppression of the HMGI(Y) and HMGI-C proteins and prevented the neoplastic transformation induced by the myeloproliferative sarcoma virus (MPSV) or by the Kirsten murine sarcoma virus (KiMSV) (17). The same HMGI-C antisense construct caused a temporary reversion of the neoplastic morphology of rat thyroid malignant cells (ref. 17; M. T. Berlingieri and A.F., unpublished results). Therefore, suppression of HMGI proteins with the antisense technique may be envisaged as a treatment strategy for human malignant neoplasias.

We have generated a recombinant adenovirus carrying the HMGI(Y) sequences in an antisense orientation, and we demonstrate that this vector suppresses HMGI(Y) protein synthesis and induces cell death in two human thyroid anaplastic carcinoma cell lines, but not in normal thyroid cells. *In vivo* we demonstrate that injection of the Ad-Yas virus into athymic mice suppresses the growth of xenograft tumors induced by anaplastic thyroid carcinoma cells.

Materials and Methods

Preparation of the Recombinant Adenoviruses. The mouse HMGI(Y) cDNA of 1,500 bp was inserted, as a *Hin*dIII fragment, in sense and antisense orientation into pac-CMVpLpa to generate the pac-CMV-HMGI-Ys and pac-CMV-HMGI-Yas constructs, respectively. They were cotransfected with pJM17 into the human embryonic kidney 293 cell line (American Type Culture Collection) to generate the Ad5CMV-HMGI(Y)s (Ad-Ys) and the Ad5CMV-HMGI(Y)as (Ad-Yas) viruses. Viral stocks were expanded in 293 cells, which were harvested 36–40 h after infection and lysed. Virus titer was determined by plaque-forming units (pfu) on the 293 cells. Viral stocks were aliquoted and stored at −80°C. The AdCMV-lacZ (Ad-lacZ) vector (Quantum Biotechnology, Montreal) also was used as a control.

Cell Lines. The ARO (18) and FB-1 (19) cells were derived from human thyroid anaplastic carcinomas. LoVo, GEO, and SW620 were derived from human colon carcinomas. A549 and H1355 were derived from human non-small-cell lung carcinomas. MCF-7 and MDA468 were derived from human breast carcinomas. All these cells were grown in DMEM containing 10% FCS (GIBCO/BRL). The PC Cl 3 cells are normal thyroid cells of Fischer rat origin. The PC MPSV cells are PC Cl 3 cells infected with the MPSV carrying the v-*mos* oncogene (20). These cells were grown in Ham's F-12 medium, Coon's modification (Sigma), supplemented with 5% calf serum (GIBCO/BRL) and a mix containing six growth factors (6H) comprising 10 nM thyroid-stimulating hormone, 10 nM hydrocortisone, 100 nM insulin, 5 μg/ml transferrin, 5 nM somatostatin, and 20 μg/ml glycyl-histidyl-lysine. HTC 2 cells are described elsewhere (21).

RNA Isolation and Reverse Transcription–PCR (RT-PCR) Analysis of the Expression of the HMGI(Y) Antisense Construct. Total RNA was extracted with the RNAfast Isolation System (Molecular System, San Diego). A previously described strand-specific RT-PCR procedure (17) was used to detect the antisense HMGI(Y)-specific sequences. The primers corresponded to nucleotides 68–88 and 141–161 of HMGI(Y) cDNA. Expression of the rat glyceraldehyde-3-phosphate dehydrogenase gene served as an internal control for the amount of cDNA in the PCRs, by amplification of a 430-bp cDNA fragment in the presence of the following oligonucleotide primers: 5′-TCACCATCTTCCAGGAGCGAG-3′ (forward) and 5′-ACAGCCTTGGCAGCACCAGT-3′ (reverse). To verify that

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Abbreviations: HMGI, high mobility group I; MPSV, myeloproliferative sarcoma virus; pfu, plaque-forming unit(s); RT-PCR, reverse transcription–PCR; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP end labeling.

§To whom reprint requests should be addressed at: Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli, 80131 Naples, Italy. E-mail: afusco@napoli.com.

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RNA samples were not contaminated by DNA, we obtained negative controls by running PCRs on samples that were not reverse-transcribed, but otherwise processed identically to the samples used for the experiment.

Immunoblotting Analysis. Cells were washed once in cold PBS and lysed in a buffer containing 50 mM Hepes (pH 7.5), 1% (vol/vol) Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM PMSF, and 0.2 mg each of aprotinin and leupeptin per ml. Lysates were clarified by centrifugation at 10,000 $\times$ *g* for 15 min, and the supernatant was stored at -70°C. Protein concentration was estimated by a modified Bradford assay (Bio-Rad). Total proteins were separated by 15% SDS/PAGE and transferred to poly(vinylidene difluoride) membranes (Immobilon-P; Millipore). Membranes were blocked with 5% nonfat milk proteins and incubated with polyclonal antibodies raised against a synthetic peptide located in the NH_2-terminal region of the HMGI(Y) protein (10) at a dilution of 1:1,000. Bound antibodies were detected by the appropriate secondary antibodies and revealed with the Amersham Pharmacia enhanced chemiluminescence system.

Viability and Cell Growth Assay. Cells (5×10^4) were seeded in a six-multiwell system in a medium culture containing 10% calf serum. After 24 h, cells were exposed to Ad-Yas, Ad-Ys, or Ad-lacZ (10 pfu per cell). After a 90-min incubation at 37°C, the virus was removed and the incubation continued in fresh medium at 37°C. Cells were trypsinized each day and counted by using a hemocytometer. For the evaluation of the cytotoxic effects of the Ad-Yas virus, cells were seeded in 96-well plates (1,000–2,000 per well); 24 h later, different doses of adenovirus were added to the incubation medium. The cells were incubated for 7 days in the presence of adenovirus before fixation with 10% trichloroacetic acid and staining with 0.4% sulforhodamine B in 1% acetic acid (22). The bound dye was solubilized in 100–200 ml of 10 mM unbuffered Tris solution, and the optical density was determined at 540 nm in a microplate reader (Bio-Rad). The percentage survival rates of cells exposed to adenovirus vectors was calculated by assuming the survival rate of untreated cells to be 100%.

Terminal Deoxynucleotidyltransferase-Mediated dUTP End Labeling (TUNEL) Assay. For the TUNEL assay we used the *In Situ* Cell Death Detection Kit (Boehringer Mannheim), following the manufacturer's instructions. Briefly, the air-dried cells were fixed with a freshly prepared paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. The slides then were rinsed with PBS and incubated in permeabilization solution (0.1% Triton X-100/0.1% sodium citrate) for 2 min on ice. The slides then were rinsed twice with PBS, and 50 μl of TUNEL reaction mixture was added. After a 30-min incubation at 37°C, substrate solution was added. After 10 min at room temperature the slides were mounted under glass coverslips and examined under the light microscope.

Flow-Cytometric Analysis. ARO and FB-1 cells infected with Ad-Yas, Ad-Ys, or Ad-lacZ were analyzed for DNA content as described previously (23). Cells were collected and washed in PBS. DNA was stained with propidium iodide (50 μg/ml) and analyzed with a FACScan flow cytometer (Becton Dickinson) interfaced with a Hewlett–Packard computer. Cell cycle data analysis was performed by the CELL-FIT program (Becton Dickinson).

Tumorigenicity Assay. All experiments were performed in 6-week-old male athymic mice (Charles River Breeding Laboratories). ARO cells (2×10^6) untreated or transduced previously with Ad-Yas (10 pfu per cell), Ad-Ys (10 pfu per cell), or Ad-lacZ (10 pfu per cell) were injected into the flank of athymic mice. Another group of animals was injected with ARO cells (2×10^6) s.c. Three days later, Ad-Yas or Ad-lacZ was injected in the inoculation region or i.p. In another set of experiments, ARO cells (2×10^6) were injected into athymic mice, and 20 days later, when a tumor became visible, Ad-Yas or the Ad-lacZ was injected into the tumoral mass or i.p. weekly. For the intratumoral treatment we used a dose of 5×10^7 pfu, whereas for the i.p. treatment, 5×10^8 pfu were injected.

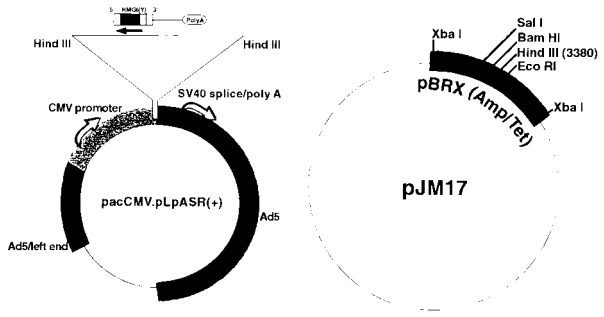

Fig. 1. Generation of the Ad-Yas virus carrying the HMGI(Y) gene in an antisense orientation. Homologous recombination between shuttle vector pac-CMVpLpA containing an expression cassette of mouse HMGI-Y cDNA in an antisense orientation (HMGI-Yas) (*Left*) and the adenovirus genome cloned in plasmid pJM17 (*Right*) generated an adenovirus clone in which the adenovirus E1A region was replaced by the HMGI(Y) cDNA expression cassette.

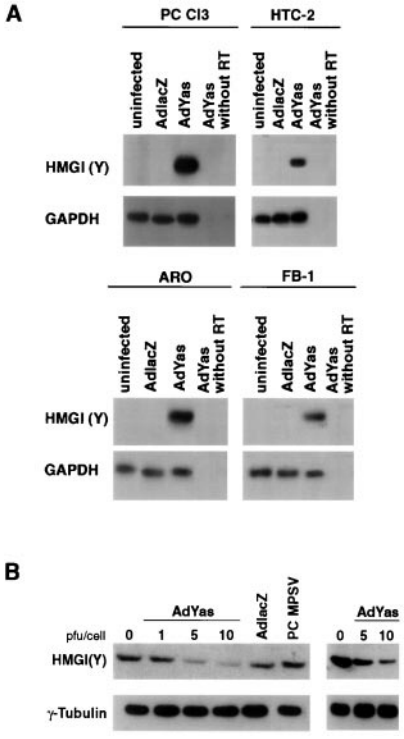

Fig. 2. Expression of the HMGI(Y) antisense sequences and inhibition of HMGI(Y) protein synthesis in human thyroid carcinoma cell lines by Ad-Yas. (*A*) Strand-specific RT-PCR assay for the HMGI(Y) antisense sequences in the PC Cl 3, HTC 2, ARO, and FB-1 cells infected with Ad-Yas (10 pfu per cell) and Ad-lacZ (10 pfu per cell). No amplification was obtained when ARO and FB-1 extracted from Ad-Yas-infected cells were analyzed without reverse transcriptase. (*B*) Western blot analysis of the HMGI(Y) proteins in ARO (*Left*) and FB-1 (*Right*) cells infected with Ad-Yas at different concentrations, as indicated, and Ad-lacZ. The PC MPSV cells are PC Cl 3 cells infected with the MPSV carrying the v-*mos* oncogene (20). These cells were used as a positive control. Analysis of γ-tubulin protein was performed as a control for protein loading.

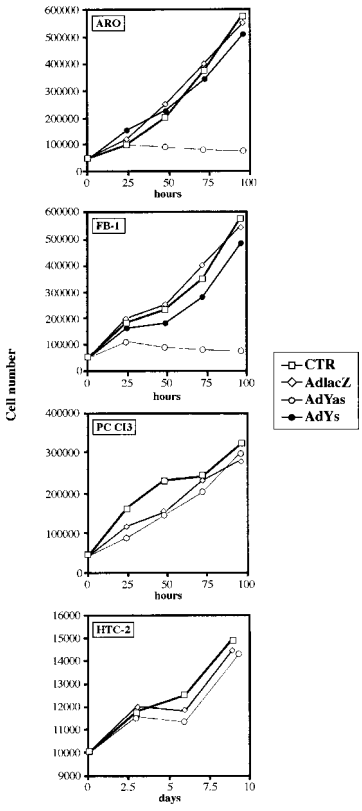

Fig. 3. Cell growth of Ad-Yas-infected cells. ARO, FB-1, PC Cl 3, and HTC 2 cells were plated (50,000 per well) and, the day after, were exposed to 10 pfu per cell of either Ad-Yas, Ad-Ys, or Ad-lacZ and harvested daily for cell counts.

Tumor diameters were measured with calipers every other day until the animals were killed. Tumor volumes (V) were calculated by the rotational ellipsoid formula: $V = A \times B^2/2$ (A = axial diameter; B = rotational diameter). None of the mice showed signs of wasting or other evident signs of toxicity. All mice were maintained at the Dipartimento di Biologia e Patologia Animal Facility, and all animal studies were conducted in accordance with the Italian regulation for experimentations on animals.

Immunohistochemistry. For the immunohistochemical analysis, 5- to 6-μm-thick paraffin sections were deparaffinized and then placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and then washed in PBS before immunoperoxidase staining. Slides then were incubated overnight at 4°C in a humidified chamber, with the primary antibodies diluted 1:100 in PBS and subsequently incubated, first with biotinylated goat anti-rabbit IgG for 20 min (Vectastain ABC kits; Vector Laboratories) and then with premixed reagent ABC (Vector Laboratories) for 20 min. The immunostaining was performed by incubating slides in diaminobenzidine (DAB; Dako) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS, pH 7.6, for 5 min, and after chromogen development, slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips by using a permanent mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a Zeiss photo system. The antibodies used in this study were goat polyclonal antibodies (Virostat) raised against the hexon from adenovirus type 2.

Results

Generation of an Adenovirus Construct Carrying the HMGI(Y) Gene in an Antisense Orientation. We generated a replication-defective adenovirus carrying the HMGI(Y) gene in an antisense (Ad-Yas) orientation by homologous recombination between the shuttle vector pac-CMVpLpA containing an expression cassette of mouse HMGI(Y) cDNA in antisense orientation and the adenovirus genome cloned in plasmid pJM17 (Fig. 1). By the same procedure, we generated an adenovirus carrying the HMGI(Y) gene in sense orientation (Ad-Ys).

Table 1. Cytotoxic effect of Ad-Yas on neoplastic cells

Tissue origin	Cell line	HMGI(Y) expression*	IC_{50} Ad-Yas, pfu/cell†
Rat thyroid normal cells	PC Cl 3	ND	>1,000
Rat thyroid transformed cells	PC MPSV	High	10
Human thyroid normal cells	HTC-2	ND	>1,000
Human thyroid cancer cells	ARO	High	2.6
	FB-1	High	3
Human colon cancer cells	LoVo	High	1
	GEO	High	12.5
	SW620	High	5
Human lung cancer cells	A549	High	9
	H1355	High	4.5
Human breast cancer cells	MCF-7	High	25
	MDA468	High	13

*The level of endogenous HMGI(Y) in each cell line was determined by immunoblotting analysis. ND, not detectable.
†Values are estimated from the cell-killing experiment described in the text.

Infection of Thyroid Carcinoma Cell Lines with Ad-Yas Suppresses HMGI(Y) Protein Synthesis. The human anaplastic thyroid carcinoma is an appropriate target for innovative gene therapy because it is one of the most aggressive human neoplasias and is resistant to any known therapeutic approach. Therefore, we examined the effect of Ad-Yas on two cell lines, ARO and FB-1 (18, 19), both of which originate from human thyroid anaplastic carcinomas and express high HMGI(Y) levels (10, 19).

We first demonstrated, by strand-specific RT-PCR assay, the

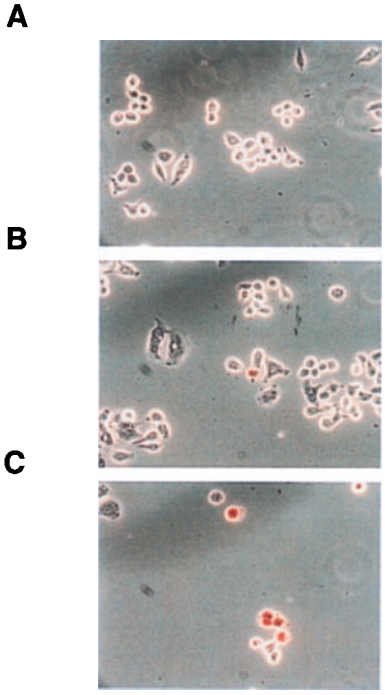

Fig. 4. TUNEL assay of the Ad-Yas-infected ARO cells. (*A*) Untreated ARO cells. (*B*) ARO cells infected with Ad-lacZ (10 pfu per cell). (*C*) ARO cells infected with Ad-Yas (10 pfu per cell).

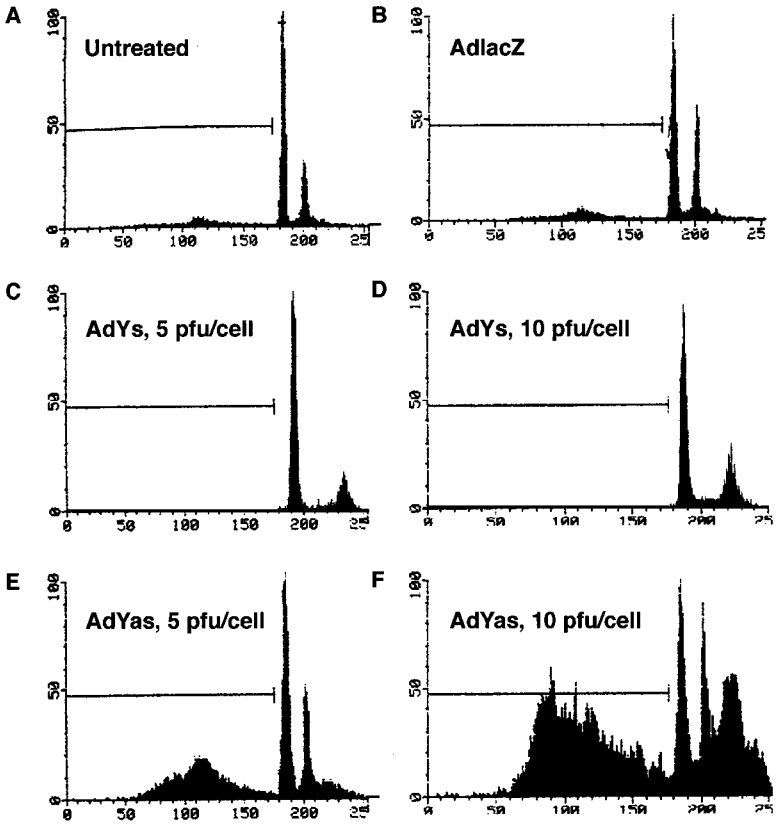

Fig. 5. Flow cytometric analysis of the Ad-Yas-infected ARO cells. The DNA content of ARO cells was analyzed by flow cytometry after propidium iodine staining. Cells either were treated with PBS (*A*) or infected with Ad-lacZ 10 pfu per cell (*B*), Ad-Ys (5 and 10 pfu per cell) (*C* and *D*, respectively), or Ad-Yas (5 and 10 pfu per cell) (*E* and *F*, respectively).

expression of the HMGI(Y) antisense construct in normal and malignant thyroid cells infected with Ad-Yas (Fig. 2*A*). Subsequently, Western blot analysis showed a significant reduction of HMGI(Y) protein levels in both ARO and FB-1 cells infected with Ad-Yas (Fig. 2*B*). Densitometric analysis revealed a 5-, 10-, and 20-fold reduction in HMGI(Y) protein levels in the ARO cells treated with 1, 5, and 10 pfu per cell Ad-Yas (Fig. 2 *Left*), respectively. Analogous results were obtained with FB-1 cells (Fig. 2 *Right*). Conversely, Ad-lacZ infection did not affect HMGI(Y) levels in either ARO (Fig. 2 *Left*) or FB-1 cells (data not shown). No changes in γ-tubulin protein levels were observed in the cell lines infected with the same adenoviruses, which indicates that equal amounts of protein were loaded and that no nonspecific inhibition of protein synthesis occurred in the infected cells.

Cytotoxic Effect of Ad-Yas on Neoplastic Cells. To study the effect of Ad-Yas on cell growth we plated PC Cl 3, normal rat thyroid cells, HTC 2 cells, normal human thyroid cells, and human ARO and FB-1 cells (50,000 per well), and the day after we exposed them to 10 pfu per cell of Ad-Yas, Ad-Ys, or Ad-lacZ. The treated cells were harvested daily for cell counting. As shown in Fig. 3, infection of ARO and FB-1 cells with the Ad-Yas virus significantly reduced cell number over the 4 days examined. In contrast, the growth of PC Cl 3 and HTC 2 cells, which do not express HMGI(Y) proteins (7, 16), was not affected by infection with the adenoviruses (Fig. 3) even though antisense HMGI(Y) sequences were expressed in the Ad-Yas-infected PC Cl 3 and HTC 2 cells (Fig. 2*A*).

Subsequently, ARO and FB-1 cells were exposed to increasing concentrations of Ad-Yas and Ad-lacZ for 7 days. As shown in Table 1, ARO and FB-1 cells were very sensitive to the lethal effect of Ad-Yas (IC_{50} = 2.6 and 3.0, respectively). The PC MPSV cells, which are rat thyroid cells transformed by the MPSV that show a highly malignant phenotype (19), responded like the ARO and FB-1 to Ad-Yas. Conversely, no lethal effect was observed when the PC Cl 3 and HTC 2 were infected at 1,000 pfu per cell.

Because HMGI(Y) gene overexpression has been reported in several human and experimental tumors other than thyroid, we analyzed the effects of HMGI(Y) protein suppression by Ad-Yas on three human colon carcinoma (SW620, LoVo, and GEO), two human lung carcinoma (A549 and H1355), and two human breast carcinoma (MCF-7 and MDA468) cell lines. All these carcinoma cell lines were very sensitive to the Ad-Yas virus (Table 1).

The Ad-Yas Virus Induces Apoptosis in Human Thyroid Cancer Cells. To determine the mechanism by which suppression of HMGI(Y) synthesis leads to the death of the neoplastic cells examined, Ad-Yas-infected ARO and FB-1 cells were analyzed by two apoptotic assays. TUNEL assay showed positive staining of the Ad-Yas-infected ARO (Fig. 4*C*) and FB-1 (data not shown) cells. Consistently, flow cytometric analysis revealed a shift of the DNA profile to a sub-G_1 position when ARO cells were treated with 5 and 10 pfu per cell of Ad-Yas virus (Fig. 5 *E* and *F*, respectively). Analogous results were obtained with FB-1 cells (data not shown). Conversely, neither of the cell lines infected with the Ad-lacZ virus showed positive staining by TUNEL assay (Fig. 4*B*), and neither of the cell lines infected with the Ad-lacZ or Ad-Ys virus showed changes in the cell cycle (Fig. 5 *B–D*).

Suppression of ARO Cell Line Tumorigenicity by the Ad-Yas Virus. To evaluate the efficacy of the Ad-Yas virus in inhibiting tumor growth *in vivo*, we analyzed the effect of Ad-Yas on a xenograft of ARO cells in athymic mice. We assessed the ability of the adenoviral vector to transduce *in vivo* evaluating immunohistochemically β-galactosidase activity in an ARO-induced tumor 4 days after intratumoral injection with the Ad-lacZ virus. The transduction efficiency was higher than 90% (data not shown).

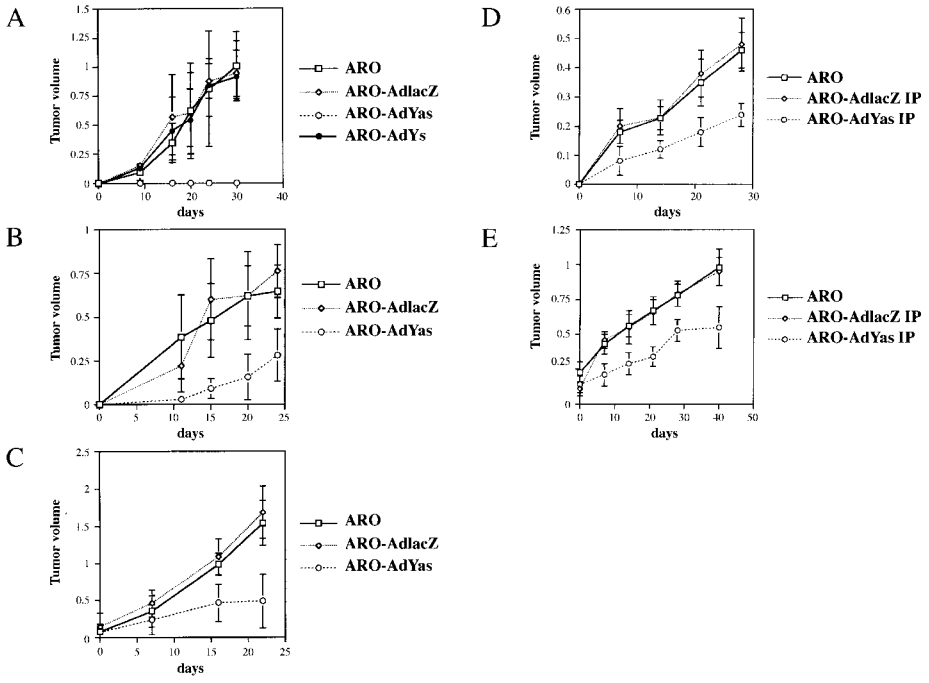

Fig. 6. Suppression of the ARO-induced tumor growth by Ad-Yas infection. (*A*) ARO cells were infected with either Ad-Yas (10 pfu per cell), Ad-Ys (10 pfu per cell), or Ad-lacZ (10 pfu per cell) and inoculated into eight athymic mice. (*B*) ARO cells (2×10^6) were inoculated s.c. in 24 athymic mice, and, 3 days later, eight mice were treated in the area of the lesion with 5×10^7 pfu of either Ad-Yas or Ad-lacZ. (*C*) ARO cells (2×10^6) were inoculated s.c. in 24 athymic mice. Twenty days later, when the tumors were detectable, eight mice were treated once a week with 5×10^7 pfu of either Ad-Yas or Ad-lacZ in the area of the lesion. (*D*) ARO cells (2×10^6) were inoculated s.c. in 90 athymic mice, and, 3 days later, 30 mice of these were treated i.p. with 5×10^8 pfu of either Ad-Yas or Ad-lacZ once a week. (*E*) ARO cells (2×10^6) were inoculated s.c. in 90 athymic mice, and, 20 days later, when the tumors became visible, 30 mice were treated i.p. with 5×10^8 pfu of either Ad-Yas or Ad-lacZ once a week.

First, the ARO cells were preadsorbed with Ad-Yas (10 pfu per cell) before injection into athymic mice. When the ARO cells were infected with Ad-Yas, no tumor was detected in seven of eight mice after 30 days (Fig. 6*A*). Conversely, all of the athymic mice injected with the untreated ARO cells and those treated with the control Ad-lacZ or Ad-Ys virus (10 pfu per cell) developed tumors in the inoculation site, with a size ranging between 0.6 and 0.9 cm^3. In addition, ARO cells were inoculated s.c. in 24 nude mice, and 3 days later 8 mice were treated in the area of the lesion with 5×10^7 pfu of either Ad-Yas or Ad-lacZ. The Ad-Yas treatment inhibited tumor growth. In fact, the average tumor size was 4-fold less in Ad-Yas-treated mice than in untreated and Ad-lacZ-treated mice (Fig. 6*B*).

To investigate whether Ad-Yas was able to suppress the growth of an already established ARO-induced tumor, ARO cells were injected into athymic mice, and 20 days later, when tumors were clearly detectable, the Ad-Yas virus was injected (5×10^7 pfu) each week for 4 weeks in the peritumoral area. During the following 4 weeks, only a weak further increase in tumor size occurred. Conversely, the ARO-induced tumors, either untreated or treated with the control Ad-lacZ virus, greatly increased in tumor size (Fig. 6*C*). Neither control nor tumor-bearing mice showed signs of toxicity after a 30-day treatment with Ad-Yas. Interestingly, i.p. treatment of xenograft tumors with Ad-Yas showed an efficacy similar to that obtained by injecting the adenoviral construct in the peritumoral area. In fact, i.p. inoculated Ad-Yas (5×10^8 pfu) inhibited the growth of both incipient (Fig. 6*D*) and established (Fig. 6*E*) tumors. It is noteworthy that for the last two experiments (shown in Fig. 6

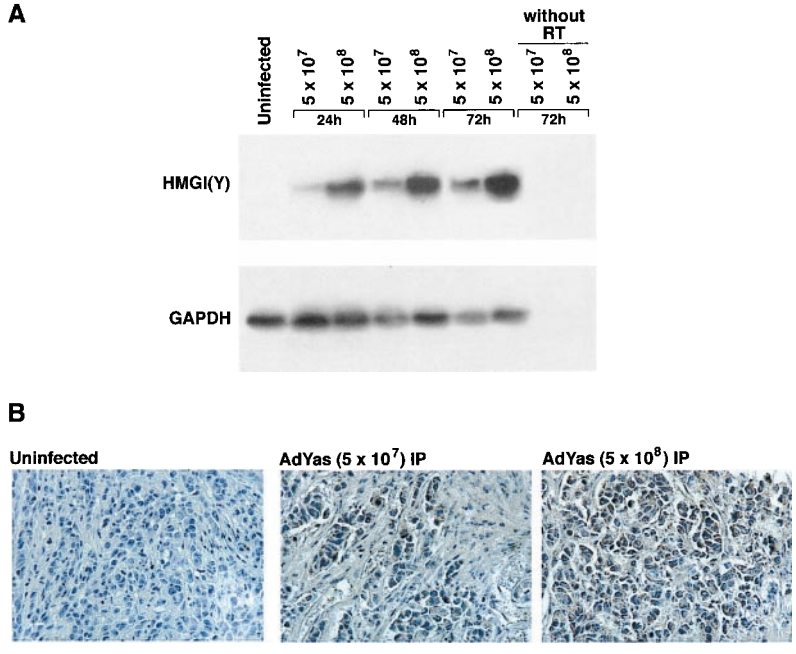

Fig. 7. (*A*) Detection of the Ad-Yas virus in the ARO xenograft tumor by RT-PCR analysis after i.p. treatment. Strand-specific RT-PCR assay for the HMGI(Y) antisense sequences. The cDNA was coamplified with glyceraldehyde-3-phosphate dehydrogenase-specific primers as an internal control. No bands were detected in non-reverse-transcribed RNAs, thereby excluding DNA contamination. (*B*) Immunohistochemical detection of the Ad-Yas virus in the xenograft tumor after i.p. treatment. Paraffin sections from tumors treated with Ad-Yas were analyzed by immunohistochemistry after 48 h by using antibodies raised against the hexon from adenovirus type 2. No immunoreactivity was observed in untreated tumors (×40). Conversely, strong immunoreactivity was observed after i.p. treatment with either 5×10^7 pfu (×40) or 5×10^8 pfu (×40).

D and *E*) 30 animals for each group were treated. To evaluate delivery of the Ad-Yas virus to the xenograft ARO-induced tumor after i.p. injection, we analyzed Ad-Yas virus mRNA-specific expression in the tumor (Fig. 7*A*). The Ad-Yas virus was detected in the tumor at 24, 48, and 72 h after i.p. injection: its expression was dose-dependent. The results of the immunohistochemical analysis (Fig. 7*B*) of adenoviral hexon protein in the xenograft tumor were consistent with the results obtained by RT-PCR and showed that the majority (about 70%) of the neoplastic cells were infected at 48 h by Ad-Yas after i.p. injection of 5×10^8 pfu.

Discussion

We have evaluated the efficacy of a cancer gene therapy based on suppression of HMGI(Y) protein synthesis. The rationale for this investigation stems from two observations: (*i*) overexpression of the HMGI(Y) gene in human and experimental tumors of the thyroid, prostate, colon, breast, lung, skin, and cervix (10–15) and (*ii*) normal rat thyroid cells carrying an HMGI-C gene antisense construct and not expressing either HMGI(Y) or HMGI-C proteins, which became resistant to the neoplastic transformation induced by MPSV or Kirsten murine sarcoma virus (17).

First, we generated an adenovirus construct (Ad-Yas) carrying the HMGI(Y) cDNA in an antisense orientation. This construct ensures efficient transfer and high expression of the antisense HMGI(Y) sequences, followed by significant inhibition of protein synthesis. Ad-Yas suppressed HMGI(Y) protein synthesis and inhibited cell growth in carcinoma cell lines deriving from human thyroid, lung, colon, and breast. Conversely, very little, if any inhibitory growth effect, was observed when normal rat and human thyroid cells were infected with the same adenovirus. The size of tumors induced in athymic mice by a human anaplastic carcinoma cell line (ARO) was reduced significantly by treatment with Ad-Yas, and tumor growth was arrested when the ARO cells were preadsorbed with the Ad-Yas before injection.

Taken together, our data demonstrate that the block of HMGI(Y) protein synthesis by Ad-Yas inhibits cell growth of neoplastic cells, both *in vitro* and *in vivo*, without interfering with the growth of normal cells. The differential expression of HMGI(Y) in neoplastic and normal cells accounts for the specificity and low toxicity of Ad-Yas. Its antineoplastic effect was documented in cells of different origin (thyroid, colon, lung, breast), which suggests that Ad-Yas may have a wide spectrum of applications.

The ARO and FB-1 cell lines, which derive from anaplastic thyroid carcinomas, were very sensitive to Ad-Yas. In particular, administration of Ad-Yas resulted in the regression of tumors induced in athymic mice by ARO cells. These results suggest that a therapy based on the block of HMGI(Y) protein synthesis might be beneficial in the treatment of human anaplastic thyroid carcinoma, which invariably leads to the death of patients in few months. In addition, because several neoplastic cell lines were sensitive to Ad-Yas, and HMGI(Y) overexpression appears to be a general feature of human malignant neoplasms, gene therapy based on inhibition of HMGI(Y) synthesis might be applicable to diverse types of cancer.

Interestingly, the i.p. treatment of xenograft tumors with Ad-Yas also was efficient in two different experimental conditions tested (incipient tumors and established tumors). Consistent with earlier studies (24, 25), i.p. injected Ad-Yas virus reaches the xenograft tumor in a dose-dependent fashion. This indicates that Ad-Yas was delivered efficiently to the xenograft tumor, thus leading to the inhibition of tumor growth.

Recent results, obtained in our laboratory, account for the inhibition of the neoplastic cell growth by suppression of the HMGI synthesis. In fact, we showed that thyroid neoplastic transformation is associated with a dramatic increase of AP-1 activity with the induction of the fra-1 gene, which is prevented by the suppression of the HMGI protein synthesis. Inhibition of Fra-1 protein synthesis significantly reduces the malignant phenotype of transformed thyroid cells (26). Therefore, interaction between HMGI(Y) proteins and AP-1 transcriptional complex may be required for the activation of genes whose expression is associated with malignant transformation. This hypothesis is consistent with lack of expression of the tumor-associated genes VEGF, collagenase I, and stromelysin, which are AP-1-dependent, in thyroid cell lines in which HMGI protein expression has been suppressed (26, 27).

It is noteworthy that HMGI(Y) seems to have an important role in cellular mechanisms other than malignant cell transformation. For instance, HMGI(Y) proteins are a prerequisite for HIV replication (28). Moreover, HMGI(Y) gene induction is a necessary step in adipocyte differentiation (R. M. Melillo, S.S., A. Stella, S. Battista, C. De Biasio, M.F., G. Vigietto, and A.F., unpublished results). These recent results suggest that HMGI(Y) protein suppression also might be a useful tool for the treatment of other human diseases.

In conclusion, suppression of the HMGI(Y) protein synthesis represents a new and promising approach to the therapy of human cancer.

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