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Effect of Adenoviral Transduction of the *Fragile Histidine Triad* Gene into Esophageal Cancer Cells[1]

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ABSTRACT

Reintroduction of a tumor suppressor gene product in cancer cells is a promising strategy for cancer gene therapy. The *fragile histidine triad* (*FHIT*) gene has been identified in a region at chromosome 3p14.2, which is deleted in many tumors, including esophageal cancer. Previous studies have shown frequent biallelic alterations of the *FHIT* gene in numerous tumors, and have demonstrated a tumor suppressor function of Fhit. We have studied the biological effects of adenoviral-*FHIT* transduction in esophageal cancer cell lines. Results showed suppression of cell growth *in vitro* in three of seven esophageal cancer cell lines, all seven of which showed abundant expression of the transgene. Adenoviral-*FHIT* expression, but not control adenoviral infections, induced caspase-dependent apoptosis in two esophageal cancer cell lines, TE14 and TE4, which express no or very little Fhit, respectively. Treatment of TE14 cells with adenoviral-*FHIT* vectors resulted in abrogation of tumorigenicity in nude mice. A third esophageal cancer cell line, TE12, without detectable endogenous Fhit, showed accumulation of cells at S to G_2-M and a small apoptotic cell fraction after adenoviral-*FHIT* transduction. Thus, adenoviral-*FHIT* expression can inhibit the growth of esophageal cancer cells, at least in part through caspase-dependent apoptosis, suggesting that adenoviral-*FHIT* infection should be explored as a therapeutic strategy.

INTRODUCTION

Human chromosome 3p is one of the chromosomal regions most frequently deleted in human tumors, including those of the lung, breast, esophagus, and bladder (1). Positional cloning of this region has led to identification of the *FHIT*[4] gene, which encodes a member of the histidine triad protein superfamily (2–5). The *FHIT* gene encompasses the *FRA3B* fragile site (2, 6) and a genomic locus, which is frequently involved in allelic loss, genomic rearrangement, and cytogenetic abnormalities in tumors (2–4). Numerous studies have shown genomic alterations at the *FHIT* locus, such as biallelic deletions, translocations, and the loss of Fhit protein expression in many human cancers (2–4), including those of the esophagus, lung, breast, cervix and bladder. Although point mutations within the *FHIT* gene are rare (7–9), deletions are extremely common (2–4), and, less frequently, methylation is involved in Fhit inactivation (10). Nucleotide sequence analysis of the *FHIT* locus in tumor cell lines has indicated that long interspersed nuclear element and long terminal repeat sequences are involved in homologous recombinations at the deletion end points in most cancers (11, 12). Presumably because *FHIT* encompasses the carcinogen-sensitive fragile region, the *FHIT* gene is susceptible to damage caused by environmental carcinogens, which leads to clonal expansion of Fhit-negative cells.

Esophageal cancer, one of the most deadly human tumors, occurs worldwide, and its incidence is increasing in the Western world (13, 14). Therapeutic approaches for esophageal cancer include not only conventional therapies, such as surgical removal and radiation treatment, but gene therapy strategies, such as the introduction of the tumor suppressor, p16/INK4 (15), the expression of *IL2*, *IL6*, and *GM-CSF* gene products (16, 17), and the transduction of the *herpes simplex virus-thymidine kinase* gene (18, 19). Previous studies have shown that Fhit expression is lost even in an early stage of esophageal carcinogenesis and have indicated a significant correlation with heavy smoking and alcohol habits (20, 21), providing the rationale for assessment of the biological effects of *FHIT* gene transduction in esophageal cancer cells.

Previous *FHIT* gene replacement experiments mainly involved stable transfectants of endogenous Fhit-negative tumor cells to assess the biological function of Fhit protein. Stable exogenous Fhit expression in Fhit-negative lung, gastric, and renal cancer cells resulted in inhibition of tumor cell growth (22–24), attributable, at least in part, to the induction of apoptosis (23). Similarly, Ji *et al.* (25) demonstrated that reintroduction of Fhit protein by adenoviral-*FHIT* gene transduction into lung and head-and-neck cancer cell lines caused apoptosis and the inhibition of tumorigenicity. Other studies have questioned the status of *FHIT* as a tumor suppressor based on observations of the tumorigenicity of stable *FHIT* transfectants (26, 27). To explore further the tumor suppressor question, we have studied the effect of transient Fhit expression after adenoviral-*FHIT* transduction of seven esophageal cancer cell lines. The results showed suppression of cancer cell growth *in vitro* in three of seven cell lines after adenoviral-*FHIT* transduction. One of three Fhit-susceptible esophageal cancer cell lines was tested for growth in nude mice. Tumorigenicity was abrogated by adenoviral-*FHIT* infection.

MATERIALS AND METHODS

Cell Culture. The cervical cancer cell line HeLa and esophageal cancer cells TE1, TE2, TE4, TE10, TE12, TE13, and TE14 were maintained in RPMI 1640 with 10% fetal bovine serum. SABE cells were obtained and cultured as recommended (Clonetics, Walkersville, MD). The following caspase inhibitors were obtained from Calbiochem (San Diego, CA): (*a*) Z-VAD-fmk, an inhibitor for caspases 1, 3, 4, and 7; (*b*) Z-DEVD-fmk, an inhibitor for caspases 3, 6, 7, 8, and 10; and (*c*) Z-IETD-fmk, an inhibitor for caspase 8.

Recombinant Adenoviral Vector Construction and Gene Transduction. Full-length *FHIT* cDNA was isolated from normal human placenta cDNA (Clontech) by reverse transcription-PCR strategy and confirmed by DNA sequencing (2). cDNAs for Gfp and lacZ were obtained from expression vectors (Clontech, Palo Alto, CA). Each cDNA was ligated into an adenoviral backbone DNA (Quantum, Montreal, Canada). Four adenoviral vectors, an adenoviral-*FHIT-GFP* vector that encodes two separate proteins through the internal ribosome entry site, an adenoviral-*FHIT* vector, an adenoviral-*GFP* vector, and an adenoviral-*LACZ* vector were constructed as recommended (Quantum), with minor modifications (25, 28). cDNAs are expressed under the control of a cytomegalovirus promoter (CMV5) in each vector. Briefly, each

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[4] The abbreviations used are: *FHIT*, fragile histidine triad; SABE, small airway bronchial epithelial; Z-VAD-fmk, benzyloxycarbonyl-valinyl-alaninyl-aspartyl(*O*-methyl)-fluoromethylketone; Gfp, green fluorescent protein; MOI, multiplicity of infection; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt]; Nit, nitrilase.

adenoviral vector plasmid in which cDNA was ligated was transfected into human fetal kidney 293 cells (Microbix, Toronto, Canada); after 14–21 days, homologous recombination occurred in cells, leading to plaque formation. Plaques were isolated, and supernatants were eluted to infect 293 cells in 24-well culture plates. HeLa cells were infected to check transgene expression by immunoblot analysis and confocal microscopy for Gfp. After selection of viral clones, 293 cells were infected with individual clones for each vector to develop virus stocks. The viruses were purified by CsCl gradient centrifugation. Viral titers were determined by plaque assay, absorbance measurement, and serially diluted infection of *GFP* vector aliquots prior to confocal microscopic observation.

Potential contamination with wild-type virus was monitored by PCR analysis (Quantum). Viral supernatants from infected 293 cells were treated with proteinase K (10 μg/ml) and analyzed by PCR amplification of viral DNAs. Cell pellets were treated with 1% SDS and proteinase K (10 μg/ml) before PCR amplification. DNA sequencing reactions were performed by Applied Biosystems Prism BigDye terminator reaction chemistry on a Perkin-Elmer Gene Amp PCR system 9600 and the Applied Biosystems Prism 377 DNA sequencing systems. SABE cells were infected with viral supernatants and analyzed by flow cytometry to confirm that vectors do not cause cytotoxicity (data not shown). A previous study showed that adenoviral-*FHIT* expression did not cause apoptosis nor alter cell growth in normal human bronchial epithelial cells (25).

Adenoviral infection was performed with 3×10^5 cells, which had been cultured for 24 h in six-well culture plates. Cells were incubated with adenoviral aliquots at a desired MOI in a 37°C CO_2 incubator for 1 h prior to the addition of culture medium (>25 × volume of viral sample).

Flow Cytometry, MTS Assay, and Cell Counting. Flow cytometry analysis was performed by standard protocols (29). Briefly, 1×10^5 cells were fixed with 70% ethanol for 10 min, incubated with RNase A, and stained with propidium iodide for flow cytometric analysis (29). MTS assay was performed with a kit (Promega, Madison, WI), as recommended by the manufacturer. For cell growth kinetics, 1×10^4 cells/well were cultured in six-well culture plates.

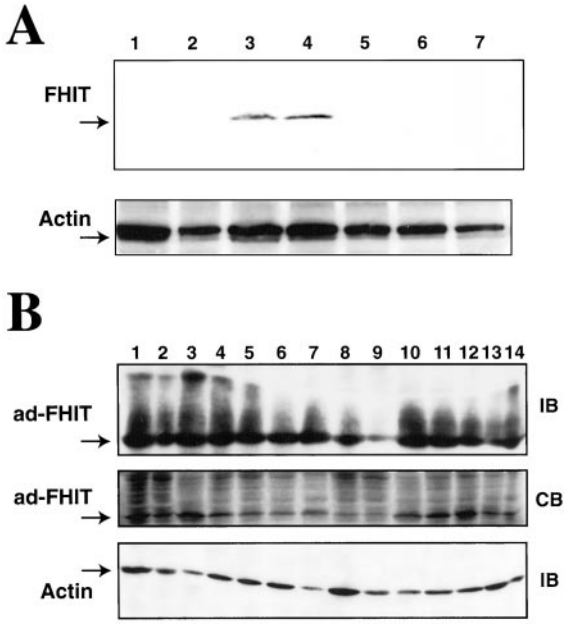

Fig. 1. Adenoviral-*FHIT* expression in esophageal cancer cell lines. *A*, immunoblot analysis of endogenous Fhit expression. Cell lysates (60 μg of protein) were subjected to SDS-PAGE. *Lane 1*, esophageal cancer TE1 cell line; *Lane 2*, TE2; *Lane 3*, TE4; *Lane 4*, TE10; *Lane 5*, TE12; *Lane 6*, TE13; *Lane 7*, TE14. *B*, adenoviral-*FHIT* expression. Expression was analyzed in cell lysates extracted at 72 h after infections at MOI 20. Cell lysates (30 μg of protein) were subjected to SDS-PAGE. Immunoblot analysis was performed with anti-Fhit antibody (*IB*, *top*) and with anti-actin antibody (*IB*, *bottom*). Coomassie Brilliant Blue staining (*CB*) is shown in the *middle*. Adenoviral-*FHIT* (*Lanes 1–7*) and adenoviral-*FHIT-GFP* (*Lanes 8–14*) vectors were used for infection of esophageal cancer TE1 (*Lanes 1* and *8*), TE2 (*Lanes 2* and *9*), TE4 (*Lanes 3* and *10*), TE10 (*Lanes 4* and *11*), TE12 (*Lanes 5* and *12*), TE13 (*Lanes 6* and *13*), and TE14 cells (*Lanes 7* and *14*).

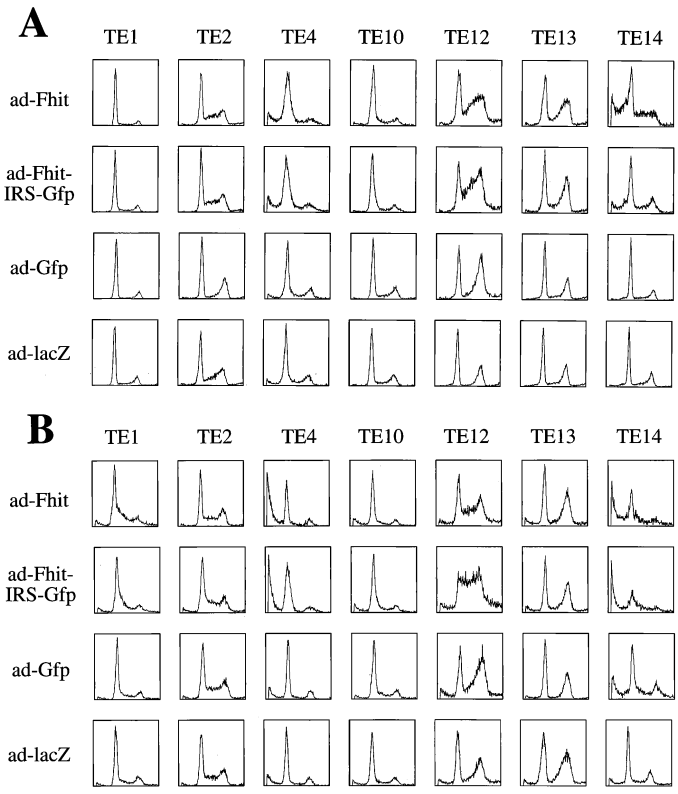

Fig. 2. Flow cytometry of infected esophageal cancer cell lines. *A* and *B*, flow cytometry analysis of esophageal cancer cell lines at 72 h (*A*) and at 5 days (*B*) after infection with adenoviral-*FHIT* (*top row* in each figure), adenoviral-*FHIT-GFP* (*second row from the top*), adenoviral-*GFP* (*third row*), and adenoviral-*LACZ* (*bottom row*) at MOI 20.

The number of cells/well was counted at indicated times in triplicate, excluding the dead cells by trypan blue staining.

Tumorigenicity. Cells were inoculated s.c. into the left dorsal region of three 6-week male BALB/c nude mice in each experimental group. The tumor volume for each mouse was determined by measuring in two directions and calculated as: tumor volume = length × $(\text{width})^2/2$ (25).

Immunoblot Analysis and Immunohistochemistry. Immunoblot analysis was performed by standard protocols (30). Briefly, cells were cultured in six-well plates and lysed for 30 min on ice in 100 μl of lysis buffer (30). Protein concentrations were determined by the Bio-Rad microassay. Cell lysates were subjected to 4–12% linear gradient SDS-PAGE and electroblotted to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% skim milk and probed with rabbit polyclonal anti-Fhit (Zymed, South San Francisco, CA), rat monoclonal anti-caspase 8 (Zymed), monoclonal anti-PARP (Clontech), goat polyclonal anti-Bid (Santa Cruz, Santa Cruz, CA), rabbit polyclonal anti-Caspase 9 (Santa Cruz), monoclonal anti-actin (Santa Cruz), monoclonal anti-lacZ (Sigma, St. Louis, MO), and monoclonal anti-Gfp antibodies (Clontech) at recommended dilutions. After probing with an appropriate secondary antibody (Amersham, Piscataway, NJ), the signal was detected by the enhanced chemiluminescence system (Amersham). Immunohistochemical analysis with antihuman Fhit antibody was performed as described (31).

RESULTS

Adenoviral Fhit Expression in Esophageal Cancer Cells *in Vitro*. Immunoblot analysis of protein from seven esophageal cancer cell lines, TE1, TE2, TE4, TE10, TE12, TE13, and TE14 showed that TE4 and TE10 cells expressed endogenous Fhit protein, whereas endogenous Fhit protein was undetectable in TE1, TE2, TE12, TE13, and TE14 cells (Fig. 1*A*). These seven esophageal cancer cell lines were infected with adenoviral-*FHIT*, adenoviral-*FHIT-GFP*, adeno-

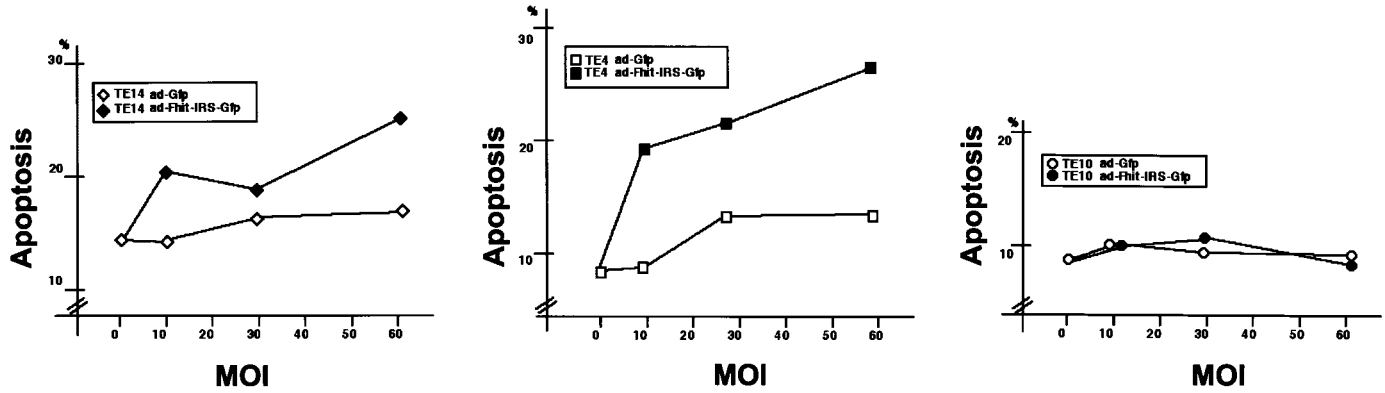

Fig. 3. Increased apoptosis of adenoviral-*FHIT*-infected esophageal cancer cell lines. Esophageal cancer cells were infected and subjected to flow cytometry analysis. Apoptotic fractions are shown for TE4, TE10, and TE14 cells infected by adenoviral-*FHIT-GFP* (*closed characters*) and adenoviral-*GFP* (*open characters*) vectors over 48 h. The *Y* axis shows the apoptotic fraction (a percentage) determined by flow cytometry, whereas the *X* axis indicates MOI.

viral-*GFP*, and adenoviral-*LACZ* vectors. Immunoblot analysis showed that, at 24 h after infection, adenoviral-*FHIT*, adenoviral-*FHIT-GFP*, adenoviral-*GFP*, and adenoviral-*LACZ* infections resulted in substantial expression of transgenes, which persisted for at least 1 week (transgene expression at 72 h after infection is shown in Fig. 1*B*). Immunoblot analysis and Coomassie Brilliant Blue staining showed that almost equal amounts of Fhit protein were expressed after infection with the same MOI of adenoviral-*FHIT* and the adenoviral-*FHIT-GFP* vectors (Fig. 1*B*).

Cell Cycle Analysis of *FHIT*-transduced Esophageal Cancer Cell Lines. Flow cytometry analysis of the seven esophageal cancer cell lines infected with adenoviral-*FHIT*, adenoviral-*FHIT-GFP*, and control vectors showed that *FHIT* transduction induced increased apoptotic cell populations in two cell lines, TE4 and TE14, whereas control vectors induced little or no apoptosis at 72 h and 5 days after infection (Fig. 2, *A* and *B*). At 72 h after infection (Fig. 2*A*) with adenoviral-*FHIT* and adenoviral-*FHIT-GFP* vectors, 23% (adenoviral-*FHIT*) and 25% (adenoviral-*FHIT-GFP*) of TE4, and 39% (*FHIT*) and 25% (*FHIT-GFP*) of TE14 cells had undergone apoptosis, whereas the fraction of apoptotic cells was increased to 53% (*FHIT*) and 42% (*FHIT-GFP*) of TE4, and 46% (*FHIT*) and 45% (*FHIT-GFP*) of TE14 cells at 5 days (Fig. 2*B*). TE4 and TE14 showed Fhit-induced apoptosis in a viral MOI-dependent manner (Fig. 3). The TE12 cell line showed cell population accumulation at the S to G_2-M phase accompanied by a small fraction of apoptotic cells at 72 h, which was much more evident at 5 days after infection with adenoviral-*FHIT* and adenoviral-*FHIT-GFP* vectors. There was little effect of infection with adenoviral-*GFP* and adenoviral-*LACZ* vectors (Fig. 2). TE1, TE2, TE10, and TE13 cells did not show obvious apoptosis nor cell cycle arrest (Fig. 2, *A* and *B*), although adenoviral Fhit protein was abundantly expressed (Fig. 1*B*). To assess another tumorigenic cell type that expresses a low level of endogenous Fhit, a cervical cancer cell, HeLa, was infected with adenoviral *FHIT* vector to determine the effect of Fhit overexpression. The results showed Fhit-

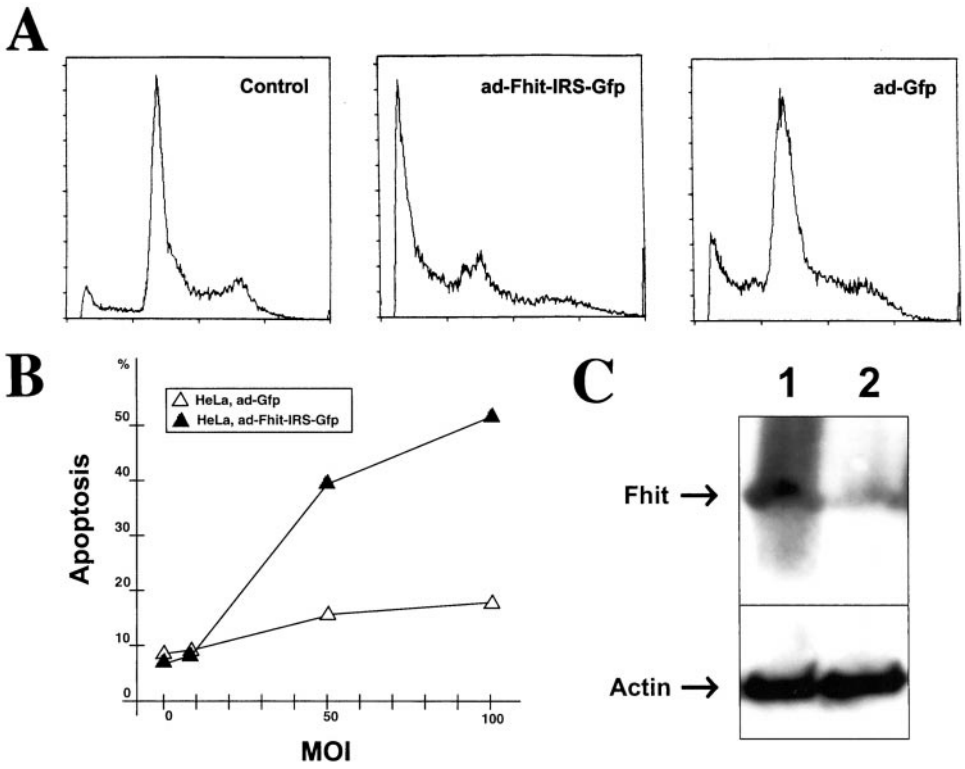

Fig. 4. Increased apoptosis of adenoviral-*FHIT*-infected HeLa cells. To assess the effect of adenoviral Fhit expression in another cell type, the cervical cancer cell line, HeLa, was infected with viral vectors. *A*, flow cytometry analysis of HeLa cells infected by adenoviral *FHIT-GFP* and adenoviral-*GFP* at MOI 50. *B*, HeLa cells were infected by adenoviral-*FHIT-GFP* (*closed characters*) and adenoviral-*GFP* (*open characters*) vectors over 48 h. *C*, the Fhit protein expression in adenoviral *FHIT-GFP* infected HeLa cells at MOI 50 (*Lane 1*) and in control HeLa cells (*Lane 2*) was analyzed by immunoblot analysis with anti-Fhit antibody (*upper lanes*) and with anti-actin antibody (*lower lanes*).

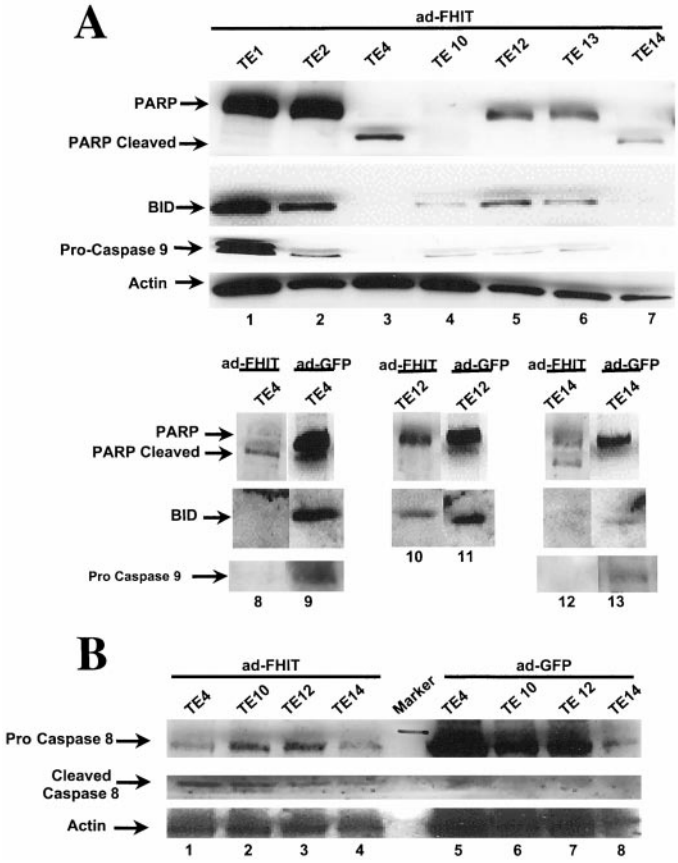

Fig. 5. Activation of proapoptotic proteins in adenoviral-*FHIT*-infected esophageal cancer cell lines. *A*, analysis of PARP, Bid, and caspase 9 in TE1 (*Lane 1*), TE2 (*Lane 2*), TE4 (*Lanes 3*, *8*, and *9*), TE10 (*Lane 4*), TE12 (*Lanes 5*, *10*, and *11*), TE13 (*Lane 6*), and TE14 (*Lanes 7*, *12*, and *13*) cells infected with adenoviral-*FHIT* (*Lanes 1-8*, *10*, and *12*) or adenoviral-*GFP* (*Lanes 9*, *11*, and *13*) at MOI 20 for 72 h. Cell lysates (50 μg of proteins) were subjected to SDS-PAGE. Immunoblot analysis was performed with PARP, Bid, caspase 9, and β-actin antibodies as indicated. Precursor forms of PARP, Bid, and procaspase 9 were cleaved in TE4 and TE14 cells. *B*, analysis of caspase 8 in TE4 (*Lanes 1* and *5*), TE10 (*Lanes 2* and *6*), TE12 (*Lanes 3* and *7*), and TE14 (*Lanes 4* and *8*) cells infected with adenoviral-*FHIT* (*Lanes 1–4*) or adenoviral-*GFP* (*Lanes 5–8*) at MOI 20 for 72 h. Activation of pro-caspase 8 resulted in a cleaved form of caspase 8. Immunoblot analysis was performed with caspase 8 and β-actin antibodies as indicated.

induced apoptosis of HeLa cells in a viral MOI-dependent manner (Fig. 4).

Analysis of Adenoviral *FHIT*-induced Apoptosis. Immunoblot analyses with antibodies against caspases 8 and 9, Bid and PARP on lysates from esophageal cancer cell lines before and after infections were compared to determine whether major mediators of apoptosis (32–34) are involved in adenoviral *FHIT*-induced cell death. We observed cleavage of Bid and caspase 9 in TE4 and TE14 after adenoviral-*FHIT* transduction, but not after adenoviral-*GFP* infection (Fig. 5). These molecules were not activated or were barely activated in TE1, 2, 10, 12, and 13 cells after adenoviral-*FHIT* induction (Fig. 5*A*). PARP was cleaved in TE4 and TE14 cells after adenoviral-*FHIT* transduction, but not after adenoviral-*GFP* infection, whereas uncleaved PARP was barely detected in TE10 after adenoviral-Gfp or adenoviral-*FHIT* infection. PARP was not activated or was barely activated in TE1, 2, 12, and 13 cells after adenoviral-*FHIT* infection (Fig. 5*A*). Caspase 8 was cleaved in all seven esophageal cancer cells after adenoviral-*FHIT* transduction but not after adenoviral-*GFP* (Fig. 5*B*). These data show involvement of caspase pathways in Fhit-induced apoptosis. To confirm this, we cultured adenoviral *FHIT*-infected TE4 and TE14 cells in medium with caspase inhibitors. When TE4 and TE14 cells were cultured with caspase inhibitors, Z-VAD-fmk, Z-DEVD-fmk, or Z-IETD-fmk, flow cytometry analysis showed that apoptotic fractions were significantly inhibited (Fig. 6, *A* and *B*). These data showed that Fhit-induced apoptosis was controlled, at least in part, by caspase-dependent pathways (34).

Cell Growth Analysis of Adenoviral *FHIT*-infected Esophageal Cancer Cell Lines *in Vitro*. MTS assay showed that *in vitro* cell growth of TE4 and TE14 cells treated with adenoviral-*FHIT* and adenoviral-*FHIT-GFP* was inhibited compared with control experiments using the adenoviral-*GFP* and the adenoviral-*LACZ* vectors (Fig. 7, *A* and *B*). Cell counts revealed that *in vitro* growth of adenoviral-*FHIT*- or adenoviral-*FHIT-GFP*-infected TE12 cells was inhibited compared with adenoviral-*GFP*- and adenoviral-*LACZ*-infected TE12 cells (Fig. 7*C*). The flow cytometry (Fig. 2) and cell growth data suggest that adenoviral-*FHIT* expression results in cell cycle arrest in TE12 cells, a response reminiscent of lung cancer cell cycle arrest and accumulation in S phase after adenoviral *FHIT* infection (25). Growth of TE1, TE2, TE10, and TE13 cells showed no significant alteration after adenoviral-*FHIT* and control vector infection (data not shown).

Tumorigenicity of Adenoviral-infected Esophageal Cancer Cells. When nude mice were inoculated with TE14, TE4, and TE12 cells, the TE14 cells, but not TE4 or TE12 cells, were tumorigenic, as reported previously (35). Nude mice were inoculated s.c. with 1×10^7 TE14 cells that had been infected *in vitro* at MOI 30 with adenoviral-*FHIT*, adenoviral-*FHIT-GFP*, adenoviral-*GFP*, and adenoviral-*LACZ* vectors and cultured for 24 h. The tumorigenicity of adenoviral-*FHIT*- or adenoviral-*FHIT-GFP*-infected TE14 cells was reduced compared with adenoviral-*GFP*- and adenoviral-*LACZ*-infected TE14 cells (Fig. 8, *A* and *B*). Immunohistochemical analysis showed that Fhit protein was abundantly expressed in TE14 cells after adenoviral-*FHIT* and adenoviral-*FHIT-GFP* infection (Fig. 8*C*).

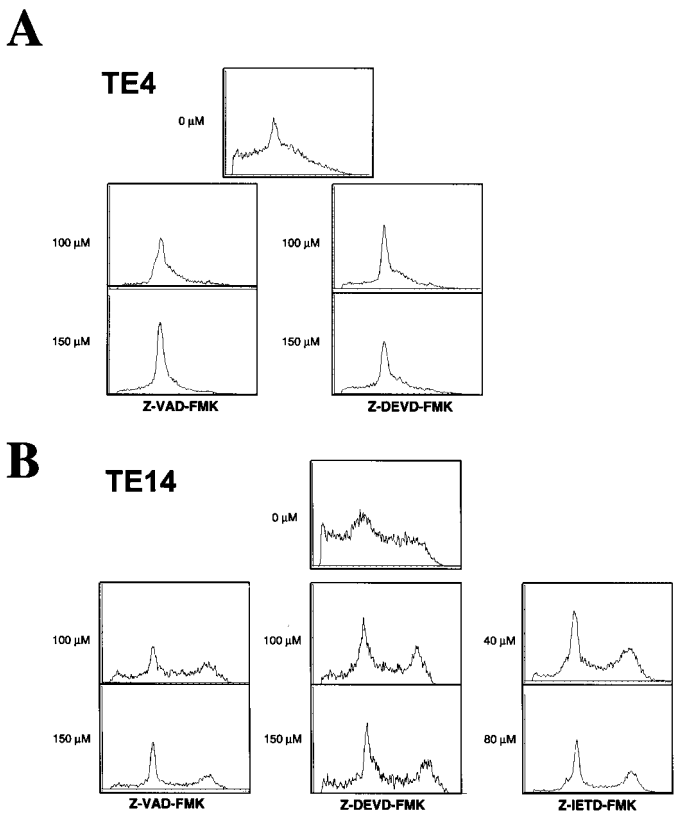

Fig. 6. Culture with caspase inhibitors in adenoviral-*FHIT*-infected esophageal cancer cell lines. TE4 (*A*) and TE14 (*B*) cells were infected with ad-*FHIT* at MOI 30 for 72 h in medium with caspase inhibitors, Z-VAD-fmk, Z-DEVD-fmk, and Z-IETD-fmk as indicated. The samples were subjected to flow cytometry analysis.

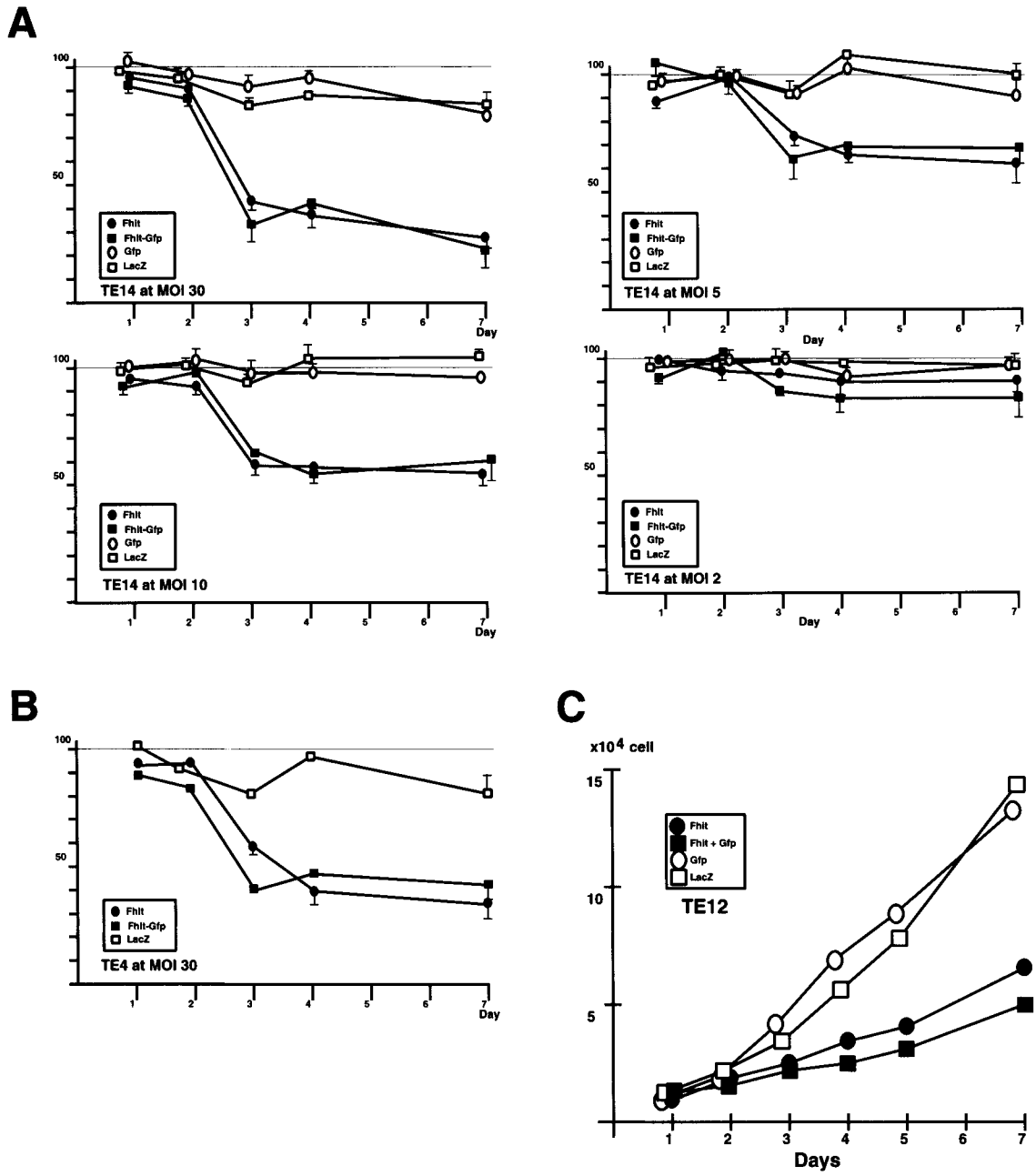

Fig. 7. *In vitro* cell growth of esophageal cancer cell lines after adenoviral-*FHIT* expression. *A* and *B*, MTS assay of TE14 (*A*) and TE4 (*B*) cells infected with adenoviral-*FHIT* (●), adenoviral-*FHIT-GFP* (■), adenoviral-*GFP* (○), and adenoviral-*LACZ* (□) vectors at MOIs 30, 10, 5, and 2 for indicated periods. Culture medium was renewed daily. Data on infection of TE4 cells at MOI 30 is shown. Results were shown as an inhibition (percentage) by comparison with noninfected control experiments. Each data point shown is an average of four independent assays. *C*, growth curve for TE12 cells; the cell number was counted by trypan blue exclusion at the indicated times after infection with adenoviral-*FHIT* (●), adenoviral-*FHIT-GFP* (■), adenoviral-*GFP* (○), and adenoviral-*LACZ* (□) vectors.

DISCUSSION

Adenoviral transduction of the *FHIT* gene product in esophageal cancer cell lines caused suppression of cell growth *in vitro* in three of seven esophageal cell lines; furthermore, after adenoviral-*FHIT* transduction, two esophageal cancer cell lines exhibited caspase-dependent apoptosis, and another showed accumulation of cells at G_2-M, with inhibition of cell growth accompanied by a small fraction of apoptotic cells. Treatment with adenoviral-*FHIT* vectors also reduced the tumorigenicity of TE14 cells *in vivo*. These data demonstrated tumor suppression by Fhit protein in three of seven, or about 40%, of esophageal cancer cell lines. Generalized toxicity of the viral vectors is ruled out because the control viruses, adenoviral-*GFP* and adenoviral-*LACZ*, did not cause alterations in cell cycle or cell growth; also adenoviral-*FHIT* expression barely affected cell cycle and cell growth in TE1, TE2, TE10, and TE13 cells, in which the transgenes were abundantly expressed. In addition, a previous study showed that adenoviral-*FHIT* overexpression (MOI 10) did not effect cell growth in normal human bronchial epithelial cells (25); similarly, flow cytometry analysis did not show significant alteration of the cell cycle in normal SABE cells after adenoviral-*FHIT* overexpression at MOI 30 (data not shown). These findings suggest additional exploration of *FHIT* transduction as a novel cancer therapeutic strategy.

The adenoviral-*FHIT* expression caused significant reduction of cell growth in three of seven cell lines, *i.e.*, endogenous Fhit(−) TE14,

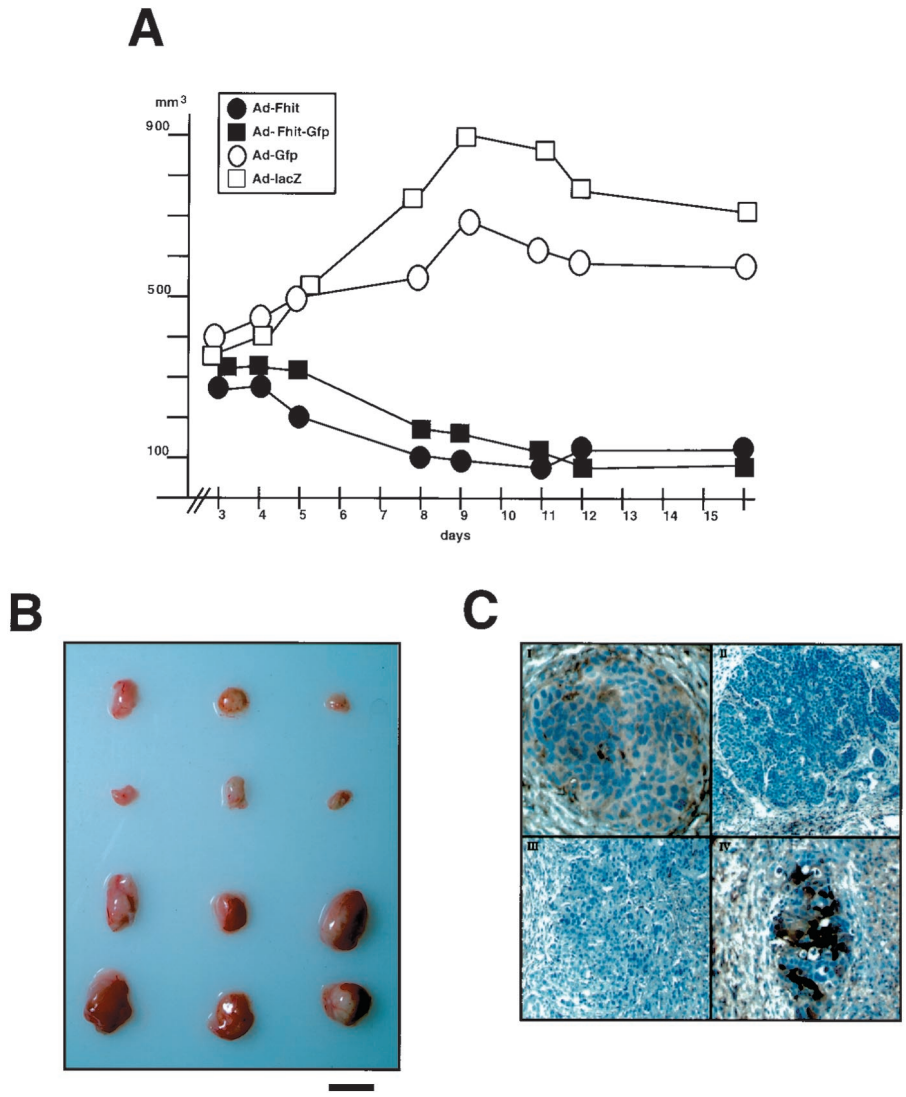

Fig. 8. Tumorigenicity of adenoviral *FHIT*-transduced TE14 cells. *A*, TE14 cells were infected by adenoviral-*FHIT* (●), adenoviral-*FHIT-GFP* (■), adenoviral-*GFP* (○), and adenoviral-*LACZ* vectors (□), as described in the text. Cells were injected s.c. into nude mice, and tumor sizes were measured. *B*, tumors formed in nude mice. Tumors were excised on day 18 after injection. The three tumors at the *top* are from TE14 cells infected with adenoviral-*FHIT*, the three in the *second row* are from TE14 cells infected with adenoviral-*FHIT-GFP*, the three in the *third row* are from TE14 cells infected with adenoviral-*GFP*, and the three in the *bottom row* are from TE14 cells infected with adenoviral-*LACZ*. Scale bar, 10 mm. *C*, immunohistochemical analysis. Excised tumor tissues were stained with antihuman Fhit antibody. *I*, tumor infected with adenoviral-*FHIT* (*upper left*; ×400); *II*, infected with adenoviral-*LACZ* (*upper right*; ×250); *III*, infected with adenoviral-*GFP* (*lower left*; ×250); *IV*, infected with adenoviral-*FHIT-GFP* (*lower right*; ×400).

Fhit(−) TE12, and Fhit(+) TE4 cells. These findings indicate that susceptibility to apoptosis or cell growth inhibition is not restricted to cancer cells with a complete loss of Fhit expression. Recent analysis with stable transfectants of renal carcinoma cells showed that susceptibility to suppression by exogenous Fhit expression is dependent on the type of cell and is not restricted to cancer cells without endogenous Fhit (24), which is compatible with the present observation. Two studies have reported that HeLa cells stably expressing exogenous Fhit showed no significant alteration in cell growth (26, 27). When we expressed the Fhit protein in HeLa cells by adenoviral-*FHIT* or adenoviral-*FHIT-GFP* infection, HeLa cells showed marked apoptosis in each experiment but not in a control experiment with *GFP* vectors. This observation suggests that the threshold of Fhit expression necessary for biological effect may differ in individual cell types.

However, the esophageal cancer cell lines in this study all expressed very high levels of exogenous Fhit protein after infection; and yet four of these cancer cell lines were insensitive to *FHIT* overexpression. One explanation might be that another gene(s) or protein(s) in the Fhit pathway has been lost or inactivated in these cancer cells. Previous studies have shown that *FHIT* homologues are encoded as fusion proteins with Nit in *Drosophila melanogaster* and in *Caenorhabditis elegans* (5, 36), suggesting that the human Nit might act in the Fhit pathway. We performed immunoblot analysis with anti-Nit antiserum, and observed that all seven esophageal cancer cells express the Nit protein (data not shown). Several observations implicate Fhit in proapoptotic pathways. Proapoptotic molecules, such as caspase 9 and Bid (34) were cleaved in both TE4 and TE14 cells, but were not or were barely cleaved in the other five cell lines after adenoviral-*FHIT* transduction. Caspase 8 was cleaved in all seven esophageal cancer cells specifically after adenoviral-*FHIT* transduction, suggesting that caspase 8 is downstream of Fhit in a signaling pathway in all of the esophageal cancer cells. When adenoviral-*FHIT*-infected TE4 and TE14 cells were cultured in medium with individual caspase inhibitors, protection from apoptosis was observed in each, suggesting that to fully execute the Fhit-induced apoptosis requires both initiators, caspases 8 and 9 (34). Although no significant changes were observed in Bcl-2 and Bcl-X_L expression in Fhit-associated apoptosis (data not shown), Bid was cleaved in both TE4 and TE14 cells after adenoviral-Fhit transduction, suggesting that Bid activation was also required for the onset of apoptosis. These data prompt us to speculate that adenoviral-*FHIT* transduction results in activation, not only of the mitochondrial pathway, but also of the caspase 8 pathway, possibly amplified through Bid cleavage (37); because caspase 8 is activated by *FHIT* overexpression in all seven cell lines, caspase 8 activation may be downstream of Fhit but upstream of caspase 9 activation and Bid and PARP cleavage.

Several recent studies have shown that endogenous Fhit expression is altered not only in advanced esophageal carcinomas but even in

precarcinomatous lesions (20, 21), and *in vivo* experiments have demonstrated that inactivation of one *Fhit* allele in recombinant mice resulted in a much higher susceptibility to carcinogen-induced esophageal/forestomach cancer (38). These observations suggest that additional studies should include investigation of the biological significance of Fhit function in the early stages of esophageal cancer and other environmental carcinogen-associated cancers.

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