The tumor-suppressor gene FHIT is involved in the regulation of apoptosis and in cell cycle control

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ABSTRACT Alteration of the FHIT (fragile histidine triad) gene occurs as an early and frequent event in lung carcinogenesis. FHIT gene transfer into lung cancer cell line H460 lacking Fhit protein expression resulted in reversion of tumorigenicity. To gain insight into the biological function of FHIT, we compared the H460 cell line with its Fhit transfectants (H460/FHit). A significant inhibition of cell growth was observed in H460/FHit cells. The analysis of apoptosis by in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling revealed a high rate of apoptosis-induced DNA strand breaks in stable clones. In situ results were confirmed by FACSscan analysis that showed an apoptotic rate of 44–47% compared with a 15% level in the control H460 cells. Analysis of cell cycle-phase distribution indicated a G1 arrest and the presence of a sub-G1 peak in the stable clones. No significant changes in Bcl2, BclX, and Bax protein expression levels were observed in the transfected clones as compared with the control H460 cells whereas a 2-fold increase in Bak protein levels was noticed. An increased level of p21Waf protein paralleled by an up-regulation of p21Waf transcripts also was found in Fhit-expressing clones isolated after transfection of various epithelial cell lines carrying inactivated endogenous Fhit, show reduced colony-forming efficiency in vitro and inhibition of tumor development in nude mice, indicating that FHIT acts as a tumor-suppressor gene (7). The FHIT protein is a diadenosine triphosphate (Ap3A) hydrolase belonging to the histidine triad superfamily (HIT) of nucleotide-binding proteins (8). Our observation that the His(96)Asn mutant, lacking hydrolytic activity, still inhibits tumor formation in vivo (7) suggests that the tumor-suppressing function of FHIT is not related to catalysis of nucleotide substrates. However, the biological mechanism of FHIT activity and the cellular pathways associated with its tumor-suppressor function are not known. Crystalllographic studies suggested that Ap3A nucleotide binding is crucial for Fhit biological activity and that enzyme–substrate complexes may be a signaling form (9). Interestingly, it has been reported that apoptosis in human cultured cells is associated with a decrease of free Ap3A levels (10).

To study a possible involvement of FHIT in cell growth control and apoptosis, we focused on the large cell lung cancer cell line H460 and its Fhit-expressing clones transfected with a FHIT-FLAG expression vector under pRc-cytomegalovirus (CMV) promoter. No detectable levels of wild-type FHIT mRNA transcript and protein are present in the H460 cell line, which, therefore, represents an ideal model for testing the effect of FHIT reintroduction. However, only few stable Fhit-expressing clones could be rescued after H460 transfection with pRc-CMV/FHit, suggesting that constitutive Fhit expression counteracts cell survival in cells lacking endogenous Fhit protein (7). In contrast, in 293 adenovirus 5 transformed human kidney cells, which constitutively express Fhit, enforced Fhit overexpression does not affect cell growth, as reflected in the high yield of rescued colonies and the high number of Fhit expressers among them (7). To get insight into the biological mechanisms underlying Fhit overexpression in lung cancer cells we studied H460 Fhit-reexpressing transduced clones for proliferation, apoptosis, and cell cycle profiles. The data showed a significantly high rate of apoptosis by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays and an altered profile of cell cycle phases distribution as suggested by a G0/G1 arrest and the presence of a sub-G1 peak. These results are consistent with the possibility that the FHIT tumor-suppressor function is related to induction of apoptosis and cell cycle alteration.

MATERIALS AND METHODS

Cells. Large cell carcinoma NCI H460 cell line (American Type Culture Collection, Manassas, VA) was maintained at 37°C in a humidified atmosphere of 5% CO2 in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (HyClone).

Plasmid. Plasmid pRc-CMV-Fhit-Flag and the empty vector pRc-CMV-5.4kb have been described previously (8).

Transfections. Exponentially growing H460 cells (1.5 × 107) were resuspended in 1 ml of RPMI supplemented with 50% FBS, mixed with 50 μg of plasmid DNA, and incubated at 4°C for 15 min. Electroporation was performed with a Bio-Rad gene pulser by using a setting of 960 mF and 250 V; three pulses were applied in all experiments. Cells then were incubated on ice for 20 min and plated in RPMI supplemented with 10% FBS and 700 μg/ml G418 (gentamicin) (GIBCO/BRL). Individual G418-resistant colonies were isolated after 2 weeks of selection and expanded in the presence of G418 antibiotic.

Abbreviations, FHIT, fragile histidine triad; Ap3A, diadenosine triphosphate; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; PI, propidium iodide.

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Cell Lysate Preparation and Western Blot Analysis. Cell lysates were prepared as described (8), and Western blots were performed by using 100 µg of total protein per lane, as described previously (11). Protein samples then were electrophoresed on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose filters, and immunoblotted with the indicated antisera. Immunoreactive bands were visualized by using horseradish peroxidase-conjugated secondary antisera and enhanced chemiluminescence (Amersham). For Western blotting, we used 1 µg/ml anti-FLAG mAb, 2.5 µg/ml anti-Bak antibody (Calbiochem), 2 µg/ml anti-p21<sup>WAF1</sup> antibody (Neo-markers, Fremont, CA), a 1:2,000 dilution of anti-actin antibody (Sigma), a 1:100 dilution of anti-p53 D07 antibody, and a 1:5,000 dilution of anti-Fhit polyclonal antibody.

Analysis of DNA Fragmentation by TUNEL. In situ detection of apoptotic cells was performed on cytospin preparations as well as on adherent cells cultured on chamber slides by using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim) and following the manufacturer’s instruction. Slides then were counterstained with 4',6-diamidino-2-phenylindole. Image acquisition was performed with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) coupled with a Zeiss Axioscope fluorescent microscope and controlled by a Power Macintosh 710/800. Frames of the nuclei were taken separately by using the IPLAB SPECTRUM (Signal Analytics, Vienna, VA) software package. The images then were pseudocolored and merged by using the GENE JOIN software (12).

FACScan analysis of apoptosis was performed as follows: 10<sup>6</sup> cells per sample were fixed with 2% paraformaldehyde in PBS (10 min on ice), washed three times with TBS (50 mM Tris-HCl in saline solution, pH 7.5), permeabilized with ice-cold acetone (1 min on ice), and washed twice in TBS and once in distilled water. Staining was performed by incubating cells for 1 hr at 37°C in 25 µl (final volume) of TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein; Boehringer Mannheim). Samples then were analyzed by FACScan. Cells with fragmented DNA appeared positive. Apoptotic cells were defined on the basis of a negative control represented by cells treated with TUNEL reaction mixture without the enzyme.

Analysis of DNA Contents by Propidium Iodide (PI) Staining. PI staining was performed as described previously (13). Briefly, 10<sup>5</sup> cells were incubated overnight at 4°C in 0.2 ml of hypotonic fluorochrome solution, containing 50 mg/ml PI (Sigma), 0.1% sodium citrate (Sigma), and 0.1% Triton X-100 (Sigma). Analysis was performed with FACScan. Cells with subdiploid DNA content were considered apoptotic cells. Cell cycle distributions were analyzed by the CELL FIT software package.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted from H460 cell line and clones 2.1 and 2.3 by RNAzol B method (Tel-Test, Friendswood, TX). Twenty micrograms of total RNA was fractionated by 1% 2.2 M formaldehyde gel electrophoresis and blotted onto nylon membrane (GeneScreen Plus; DuPont) according to standard procedures (16). The probes were radiolabeled with the Prime-It Random Primer Labeling Kit (Stratagene), and the membranes were hybridized with the ExpressHyb Hybridization Solution (CLONTECH) according to the manufacturer’s instruction. cDNA probes used were (i) a 0.5-kb EcoRI fragment from pCEVneo plasmid corresponding to the full-length human WAF1/CIP1 cDNA and (ii) a 0.9-kb BamHI-HindIII fragment from pMOSBlue plasmid corresponding to the full-length human GAPDH cDNA.

RESULTS AND DISCUSSION

To investigate a possible involvement of FHIT in apoptosis, the apoptotic rate of two stable Fhit-reexpressing clones (2.1, 2.3) first was studied by in situ TUNEL staining. These two clones showed a doubling time of 40 hr vs. 20 hr of the control H460 cell line, a reduction in efficiency and size of colony formation in both liquid medium and soft agar, and a complete (2.1) or partial (2.3) suppression of tumor formation in vivo (7). A high number of condensed and fragmented nuclei from these two clones incorporated the fluorescein-labeled dUTP, thus indicating apoptosis-induced DNA strand breaks, whereas H460 control vector transfectant showed only a baseline level of apoptotic nuclei (Fig. 1A). To determine whether the cells reexpressing the Fhit protein were those undergoing apoptosis, we performed a dual labeling with fluorescent TUNEL and rhodamine-conjugated polyclonal anti-Fhit antibody on cytospin preparations of H460/FHIT and control H460 cells. This

FIG. 1. (A) In situ TUNEL staining of clone 2.1. Condensed and fragmented nuclei incorporated the fluorescein-labeled dUTP, thus indicating apoptosis-induced DNA strand breaks. (B) Double-immunofluorescent labeling of Fhit-reexpressing cells. Apoptotic nuclei are FITC-labeled. Fhit cytoplasmic expression is detected by rhodamine-conjugated polyclonal anti-Fhit antibody.
analysis demonstrated that the majority of Fhit-reexpressing cells indeed were apoptotic and showed increased DNA fragmentation (Fig. 1B). In situ results were confirmed and quantified at FACScan by using both TUNEL and PI staining. The apoptotic rate of the two clones in different flow cytometry experiments was 44 and 47%, respectively, compared with a 15% level observed in the control H460 (Fig. 2). Moreover, PI staining of these cells and FACScan analysis of cell cycle-phase distribution revealed a significant G0/G1 arrest and the presence of a sub-G1 peak. In fact, cell cycle analysis showed 53% of cells in G0/G1, 46% in S, and 1% in G2/M phases in clone 2.1 and 52% in G0/G1, 47% in S, and 1% in G2/M phases in clone 2.3 (Fig. 2). The apparent increase of S phase could be due to apoptosis-related DNA loss of cells in G2/M phase. H460 cells were 67.1% in G0/G1, 20.1% in S, and 13% in G2/M phases. Thus, the observed growth-inhibitory effect in Fhit-reexpressing cells could be related to apoptosis and cell cycle arrest.

To characterize further FHIT involvement in the apoptotic process, we looked for an Fhit-induced modulation of apoptosis-related proteins such as Bcl-2, Bcl-X, Bax, and Bak and inhibitors of cell cycle progression such as p21waf1 and p53. The expression of these proteins was probed by Western blotting with specific antibodies on lysates from transfected clones, cultured with or without FBS, and from the control H460 cell line. We noticed, in fact, an increase in Fhit protein expression after serum starvation both in Western blot and immunocytochemical assays (Fig. 3). As a control, we probed the same samples for β-actin expression and performed densitometric analyses. No significant changes in Bcl-2, Bcl-X (data not shown), and Bax expression were observed in the transfected clones as compared with the control H460 cell line (Fig. 4A and B). However, densitometric analysis showed a reproducible 2-fold increase in Bak protein levels in 2.1 and 2.3 clones (Fig. 4C).

To investigate the cause of the G0/G1 arrest observed in transfected cells, Western blots were hybridized with antibodies detecting the expression of p21waf1 and p53. A significant increase in p21waf1 protein expression was noticed in Fhit-expressing clones 2.1 and 2.3 as compared with that of the control H460 cell line (Fig. 4D). No differences in p53 amounts were observed in the same cells, suggesting a p53-independent effect (data not shown). p21waf1 protein is a universal cell cycle inhibitor that specifically binds cyclin–CDK complexes and proliferating cell nuclear antigen, acting as a potent growth inhibitor and effector of cell cycle checkpoints (16–18).

Cell Cycle

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<tr>
<th>H460</th>
<th>SubG0/G1</th>
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| 2.1  | 47%     |

| 2.3  | 44%     |

DNA content

MFI

Fig. 2. FACScans analysis of cell cycle and apoptosis. Cytofluorimetric histograms representative of different experiments are shown. Positive controls were 20,000-rad γ-irradiated cells (60–70%). MFI, mean fluorescence intensity.
Taken together, a role for FHIT as a proapoptotic factor is in agreement with the structural and biochemical studies indicating that Fhit-Ap3A complex is the active Fhit form involved in cellular signaling and with the recent studies linking the induction of apoptosis in human cultured cells to a decrease in Ap3A level (9, 10).

Loss of Fhit protein is the most frequent alteration in non-small-cell lung cancer and preinvasive lesions and is significantly higher in the tumors of smokers than in those of nonsmokers, an event independent and even more frequent than p53 overexpression in tumors and precancerous lesions (3). The overall high frequency and precocity of Fhit loss in lung carcinogenesis and its role as a proapoptotic and cell cycle prompt FHIT gene replacement as a gene therapy of early lesions accompanying lung carcinogenesis.

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FIG. 4. Western blot analysis of Bcl-2 (A), Bax (B), Bak (C), and p21waf1 (D) in the H460 cell line and Fhit transfected clones 2.1 and 2.3. Expression levels of Bak and p21waf1 proteins were normalized on actin expression in the same lane. FBS-, cells cultured without FBS.

FIG. 5. Expression of p21waf1 mRNA in the control H460 cell line (lane 1), in FHIT transfected clones 2.1 and 2.3 (lanes 2 and 3), and in the small-cell lung cancer cell line AFL, which lacks endogenous Fhit expression (lane 4). Total RNA (20 μg) was analyzed by Northern blotting, with successive hybridization to p21waf1 and GAPDH cDNA probes.