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Advances in Brief

Overexpression of the Fanconi Anemia Group C Gene (*FAC*) Protects Hematopoietic Progenitors from Death Induced by Fas-mediated Apoptosis[1]

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Abstract

Fanconi anemia is a rare, inherited disorder characterized by bone marrow failure, congenital malformations, and cancer susceptibility. The group C Fanconi anemia gene, *FAC*, identified by expression cloning methods, encodes a protein of unknown function that may be involved in the response to apoptotic stimuli. Hematopoietic progenitor cells from *Fac* knock-out mice are hypersensitive to IFN-γ, a molecule that can induce apoptosis through up-regulation of the Fas death receptor. In this study, we used FAC-overexpressing transgenic mice to examine the relationship between FAC and Fas-triggered cell death. Hematopoietic progenitors from *FAC*-transgenic mice were up to 10-fold less sensitive to the cytolytic effect of Fas-ligation. Our experiments implicate FAC in the regulation of apoptosis mediated by the Fas death receptor.

Introduction

FA,[3] a chromosomal instability disorder, can be caused by mutations in any of at least eight genetic loci, A–H (1). *FAC*, defective in the FA-C group, encodes a novel protein of unknown function (2). Constitutive overexpression of the wild-type *FAC* gene in factor-dependent progenitor cell lines was shown to suppress apoptosis induced by the withdrawal of growth factors (3). These studies suggested that the pathophysiology of FA may involve dysregulated programmed cell death. This thesis has also generally been supported by a number of studies examining mutant FA cell lines and their response to apoptotic stimuli (4, 5). According to one report, for example, transfection of the normal FAC cDNA into these mutant cells was able to suppress apoptosis, independent of p53 function (4). Apoptosis is regulated in a very complex manner, and cells can be induced to undergo apoptosis after stimulation with either physiological activators such as growth factor withdrawal or DNA damage-related inducers. Activation of death receptors such as the Fas receptor is another important signal pathway for apoptosis (6). After signaling by any of these stimuli, the cell initiates its own death through the activation of proteases (caspases) such as the interleukin-1β converting enzyme. Bcl-2 is a prototype molecule that can suppress apoptosis and prevent protease activation (7). In this study, we concentrated on determining the role of *FAC* in the response to apoptosis by death receptor activation. As a test system, we created transgenic mice overexpressing the human *FAC* cDNA (hFAC) and analyzed their hematopoietic cell response to these stimuli.

Materials and Methods

Plasmid Construction. The construct used to generate transgenic mice was subcloned from pAAV/FACC/Neo^R (8). An expression cassette excised from this plasmid after *SalI* restriction included the *FAC* cDNA trimmed of 5′ and 3′ untranslated regions, driven by the Rous sarcoma virus promoter and including SV40 polyadenylation sequences.

Transgenic Animal Production and Care. All experiments were conducted according to a protocol approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee. The DNA construct was microinjected into C57BL/6 × SJL F2 hybrid mouse eggs by DNX, Inc. (Princeton, NJ) through contract NO1-HD-0–2911. C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME) for breeding against transgenic mice. Mice were housed in pathogen-free, individual filtered cages.

DNA, RNA, and Protein Analyses. Tail DNAs were analyzed by PCR using primer pairs specific for the human *FAC* cDNA (9), 5′-AGA GCA CAG ACT ATG GTC CA-3′ and 5′-TGC AGG AGC TCT GAG GTC TGT-3′. The PCR conditions were: (*a*) denaturation at 95°C for 2 min; (*b*) step cycling at 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 35 cycles; (*c*) extension at 72°C for 8 min; and (*d*) soak at 4°C. RNA from spleen, liver, kidney, and bone marrow were isolated; and reverse transcriptase-PCR was used to analyze FAC gene expression. The reverse transcriptase conditions were as follows: (*a*) 42°C for 30 min; (*b*) 99°C for 5 min; and (*c*) 4°C for 5 min. The cDNA PCR conditions were the same as for the DNA analysis.

Liver tissues from the indicated mouse strains were washed in ice-cold PBS containing 0.5 mM phenylmethylsulfonyl fluoride. After homogenization, the protein concentration of the soluble extract was determined. Aliquots of 25 μg were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and electroblotted on polyvinylidene difluoride membranes (NEN Research Products, Boston, MA). After blocking with 5% nonfat milk in TBST buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween], the blot was incubated sequentially with an affinity-purified polyclonal antibody directed against human FAC expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase (10) and horseradish peroxidase-conjugated antirabbit secondary antibody (Life Technologies Inc., Gaithersburg, MD), washed with TBST buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween], and immunoreactive bands identified by chemiluminescence (NEN Research Products).

Hematopoietic Progenitor Assays. For experiments involving Fas ligation, 100 ng/ml Jo-2 mAb (PharMingen, San Diego, CA) was added directly to methylcellulose cultures (at the beginning of culture) containing 10 ng/ml murine SCF (R&D, Minneapolis, MN), 10 ng/ml murine IL-3 (R&D), and 2 units/ml recombinant human erythropoietin (Amgen, Thousand Oaks, CA). CFU-GM and BFU-E were counted on day 7. Some experiments included the direct addition of murine IFN-γ (Genzyme) at a concentration of 100 ng/ml and/or murine TNF-α (Research Diagnostics, Inc., Flanders, NJ) at a concentration of 100 ng/ml. Some experiments included the Jo-2 antibody, IFN-γ and

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[3] The abbreviations used are: FA, Fanconi anemia; SCF, stem cell factor; IL, interleukin; CFU, colony-forming units; CFU-GM, CFU-granulocyte-macrophage; BFU-E, burst forming units-erythroid; TNF, tumor necrosis factor; mAb, monoclonal antibody.

TNF-α, combined, all at a concentration of 100 ng/ml (saturating concentration). For each mouse, colony assays were performed in triplicate.

Lineage Depletion of Bone Marrow Cells. Single cell suspensions were prepared by flushing femurs and tibias of wild-type or transgenic mice with PBS and then expelling through a needle. Cells were incubated on ice with a cocktail of lineage-specific mAbs at a ratio of 1 μg of each antibody per 10^6 cells. The cocktail consisted of biotinylated conjugates (PharMingen) of: anti-B220, anti-MAC-1, anti-CD4, anti-CD8, anti-Gr-1, and anti-TER-119. Cells were then washed twice and mixed with streptavidin-coated magnetic beads (Dynal) at a ratio of 20 beads:cell, 2×10^7 cells per ml, and incubated with continuous rotation at 4°C. Lineage-positive cells were removed by magnetic selection. Cytofluorimetric analysis of the resultant population was performed after staining with streptavidin-allophycocyanin (PharMingen) and a phycoerythrin-conjugated anti-Sca-1 mAb (PharMingen). For liquid culture, 4000 lin- cells were cultured with SCF, IL-3, and erythropoietin at concentrations identical to those in methylcellulose culture.

DNA Ladder Assays. DNA fragmentation was measured after extraction of low molecular weight DNA from a constant number of cells. 2×10^6 cells were resuspended in 900 μl of Tris-EDTA buffer and lysed with 25 μl of 20% SDS. The high molecular weight DNA fraction was precipitated for 6 h in the presence of 1 M NaCl and pelletted by centrifugation. Fragmented DNA was then extracted, resuspended, and subjected to electrophoresis.

Results

Creation of Transgenic Mice Overexpressing Human *FAC*. First, we constructed an expression plasmid containing the hFAC cDNA, lacking 5′ and 3′ untranslated sequences, and driven by the Rous sarcoma virus promoter (Fig. 1*A*). This DNA was microinjected into C57BL/6 × SJL F2 hybrid mouse oocytes. A sensitive PCR assay using primers specific for human *FAC* was used to genotype 24 pups. Three founder animals (one female and two males) were identified and bred against C57BL/6 mice to create a transgenic colony. Transgenic progeny from these three founders were used in our subsequent experiments. No gross developmental abnormalities were noted in either the founder or the progeny mice. No histological abnormalities were noted in brain, liver, lung, heart, kidney, spleen, thymus, bone marrow, adrenal tissue, and pancreas from transgenic animals (data not shown).

Human FAC mRNA expression was confirmed by reverse transcriptase-PCR in tissues of transgenic mice (Fig. 1*A*). Specific primers were used to distinguish the human FAC transcript from the endogenous mouse transcript. Control (nontransgenic littermate) animals did not express hFAC mRNA (Fig. 1*A*). To determine the quantity of hFAC protein expressed in transgenic animals, we performed Western analysis of liver samples (in a blinded fashion). FAC protein overexpression was confirmed in transgenic mouse liver tissue when equivalent amounts of protein samples were subjected to Western blotting using a polyclonal antiserum directed against a recombinant human FAC (Fig. 1*B*). By this assay, mice expressing various amounts of both hFAC (~60 kDa) and murine Fac protein (~59 kDa polypeptide, indistinguishable in size from the human protein) could be determined and scored semiquantitatively for total FAC protein levels (intensity of the 59–60 kDa band assessed by PhosphorImaging). The expression of total FAC in extracts from transgenic mice (scored ++ to ++++) was graded relative to the expression in wild-type mice (+).

Hematopoiesis in transgenic mice was analyzed by clonogenic colony culture. Total numbers of BFU-E, CFU-erythroid, and CFU-GM were approximately equivalent between transgenic and control littermates (data not shown). Total leukocyte and erythrocyte counts of hFAC transgenic mice were also approximately equivalent to littermate controls after matching for age and sex (data not shown).

***FAC* Protects against Fas-mediated Apoptosis.** To determine the relationship between FAC and susceptibility to Fas-mediated apoptosis, we added either IFN-γ and TNF-α (the combination known to induce Fas expression) or the Jo-2 mAb against mouse Fas directly to methylcellulose culture of progenitors. The Jo-2 antibody specifically recognizes mouse Fas and can induce apoptosis and cytolysis after Fas receptor ligation. After 1 week of culture, BFU-E and CFU-GM colonies were counted and viability compared. We first determined the concentrations of IFN-γ, TNF-α, and Jo-2 antibody that inhibited colony formation (saturating concentrations). As shown in Fig. 2,

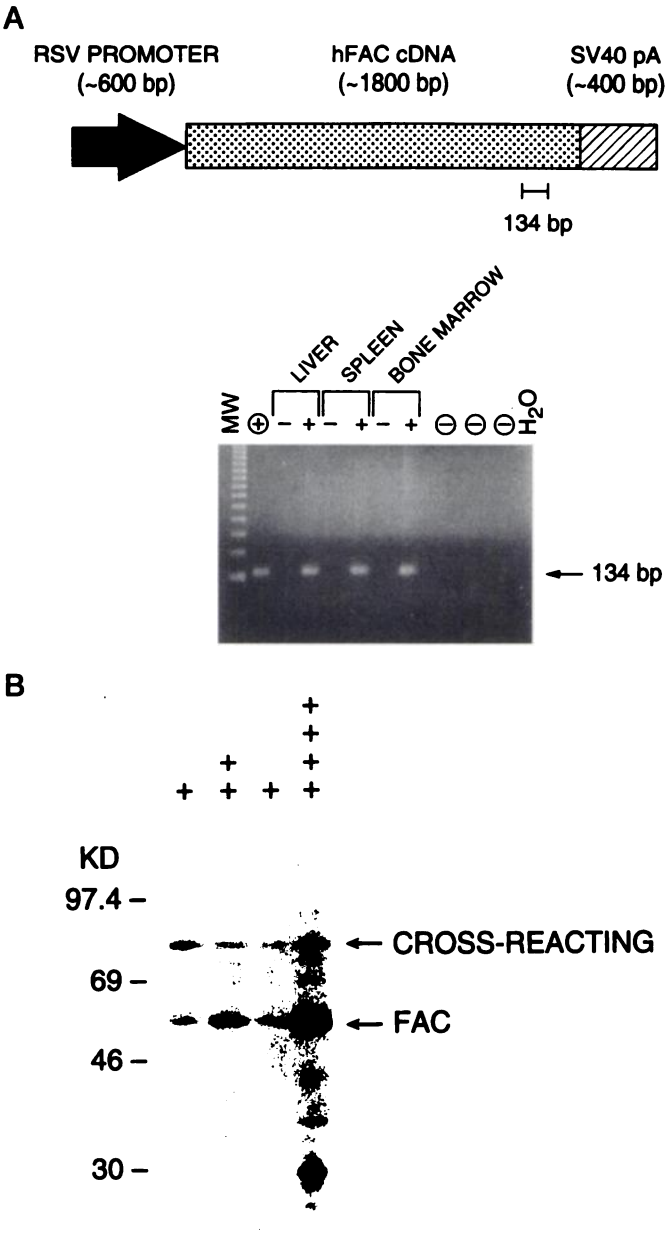

Fig. 1. *A*, human *FAC* cDNA fragment used to generate transgenic mice. Reverse transcriptase-PCR analyses demonstrated that the human FAC mRNAs were expressed in spleen, liver, and bone marrow in transgenic mouse but not in littermates (⊖, negative control). PCR included reverse transcriptase (+) and no reverse transcriptase (−) controls as well as a H_2O control. The location of the primer pair is shown schematically (*PCR fragment of 134 bp*). *B*, Western blot analysis to quantitate the amount of total FAC protein expressed in transgenic mice *versus* nontransgenic littermate controls. Using an anti-FAC protein antiserum, the combined levels of murine Fac and human FAC protein could be determined. Total FAC levels (*FAC*, with *arrow*) were scored by intensity of the 59–60 kDa band, as assessed by PhosphorImaging. The expression of total FAC in extracts from transgenic mice (++ to ++++) was graded relative to the expression in wild-type mice (+). In some of our blots, a *Cross-Reacting* band was seen from the total cell extracts.

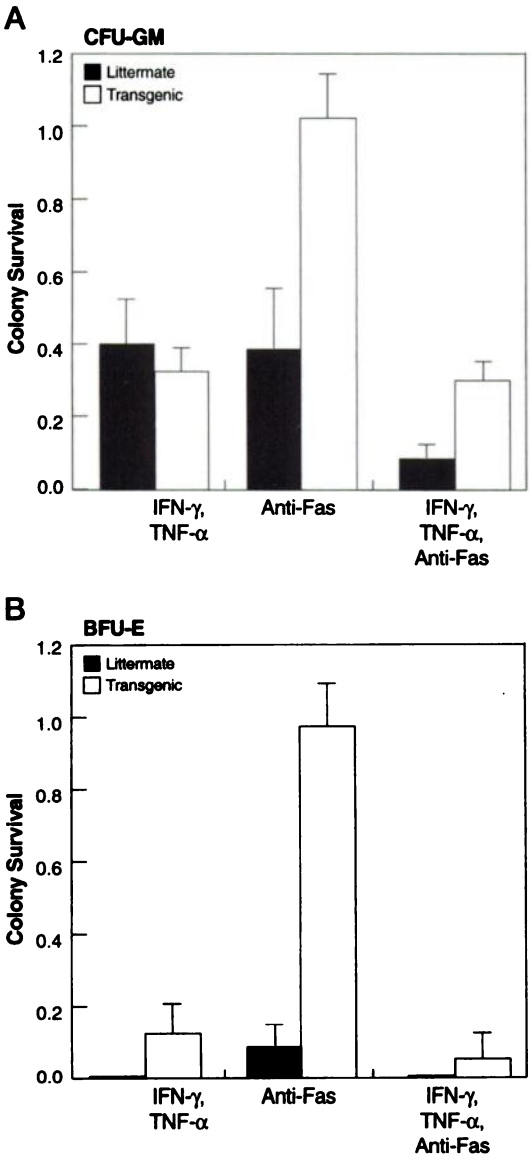

Fig. 2. *A*, resistance of *FAC* transgenic mouse CFU-GM colonies to anti-Fas antibody. *Colony survival* is expressed as compared with untreated/control colony formation given a value of 1.0 (100%). A total of 10 separate (not pooled) mouse experiments showed that there was no significant difference in inhibition—by saturating concentrations (100 ng/ml) of IFN-γ and TNF-α—of CFU-GM derived from transgenic mice or their littermate controls. However, CFU-GM from transgenic mice were 2.5 times more resistant than littermate controls to cell death induced by a saturating concentration (100 ng/ml) of Jo-2 anti-Fas mAb. In combination with IFN-γ and TNF-α, the anti-Fas antibody markedly inhibited CFU-GM formation from littermate controls and to a lesser extent CFU-GM formation from transgenic mice. *Bars*, SD. *B*, resistance of *FAC* transgenic mouse BFU-E colonies to anti-Fas antibody. *Colony survival* is expressed as compared with untreated/control colony formation given a value of 1.0 (100%). A total of 10 separate mouse experiments demonstrated that BFU-E colonies derived from hFAC transgenic mice were 10 times more resistant to cytolysis from Fas ligation than were BFU-E from littermate controls. BFU-E colonies from littermates did not grow in culture media containing IFN-γ and TNF-α, whereas, under the same conditions, BFU-E colonies from transgenic mice survived to a 10% (denoted 0.1) level of untreated controls. BFU-E from transgenic mice also were slightly more resistant to inhibition by the combination of IFN-γ, TNF-α, and anti-Fas antibody. *Bars*, SD.

FAC transgenic progenitor cells were protected against Fas-mediated cell death. CFU-GM from transgenic mice were 2.5 times more resistant than littermate controls to cell death induced by a saturating concentration of the Jo-2 antibody (Fig. 2*A*), and BFU-E from transgenic mice were 10 times more resistant (Fig. 2*B*).

To confirm that FAC protected hematopoietic progenitors from Fas-mediated apoptosis, mouse bone marrow cells were lineage-depleted using immunomagnetic bead selection. The resultant cells were depleted of lineage markers, and approximately 15% of cells were positive for the Sca-1 antigen (data not shown). After liquid culture in SCF, IL-3, and erythropoietin for 3, 5, or 6 days, the Jo-2 antibody was added to a final concentration of 1 μg/ml (supersaturating concentration) and incubated with the culture for 24 h. At that time, low-molecular weight DNA was prepared from an equivalent number (2×10^6) of viable cells from cultures either Jo-2-treated or mock. As shown in Fig. 3, fragmented DNAs in a laddered pattern, typical of apoptosis, were visualized by ethidium bromide staining in samples treated with Jo-2. The amount of degraded and fragmented DNA was variable, reflecting the different levels of apoptosis (hence, the different amounts of low molecular weight DNA in each lane). The *FAC* transgenic hematopoietic cells showed significantly less apoptosis in these assays.

Discussion

The Fas death factor is a key molecule involved in the induction of apoptosis of Fas-bearing cells (6). Fas ligand (Fas-L), which belongs to the TNF family, binds to the Fas receptor present on a variety of

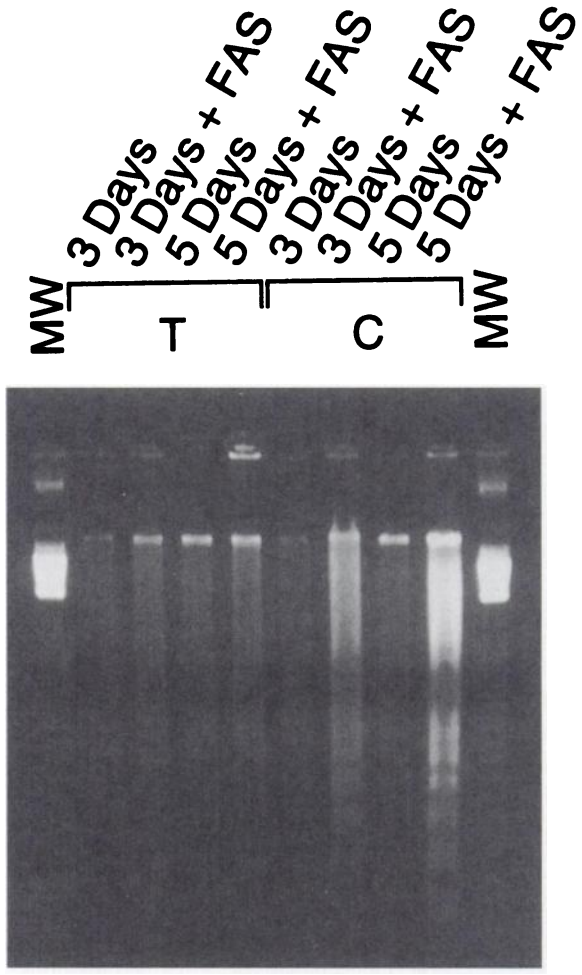

Fig. 3. Low molecular weight DNA was extracted from cultured lin- bone marrow cells from hFAC transgenic and littermate mice in the presence or absence of the Jo-2 anti-Fas antibody (*FAS*). Apoptosis could be detected by visualizing a typical DNA ladder pattern. Molecular weight standards (*MW*) are shown. Without treatment with the Jo-2 antibody, some apoptotic cells could be detected after 3- or 5-day culture of transgenic or littermate lin- cells. Addition of the Jo-2 antibody markedly increased levels of apoptosis detectable by these assays (*varying amounts of DNA in each Lane*) only in control littermate (*C*) cells. In contrast, after treatment with Jo-2, no significant increase in DNA fragmentation was detected from transgenic (*T*) cell samples.

hematopoietic cells, initiating the sequential activation of caspases. Events downstream of Fas ligation that mediate the death signal have not yet been clarified. *In vitro* studies have demonstrated that FAC overexpression can protect against apoptosis induced by growth factor withdrawal (3). Our *in vivo* experiments in this study have shown that FAC also protects hematopoietic cells from Fas-mediated cell death, suggesting a specific antiapoptotic function for wild-type FAC in a pathway downstream from these two death signals. Our results predict that inactivation of the *FAC* gene in patients may lead to higher levels of spontaneous apoptosis, a phenomenon that has in fact been reported recently (11). However, there have also been studies suggesting that a *FAC*-mutant lymphoblastoid cell line is deficient in undergoing Fas-mediated apoptosis (11). This finding initially seems inconsistent with an antiapoptotic role for FAC. However, individual cell clones may have relative degrees of resistance to apoptosis and may not reflect (or may have been selected in the presence of) widespread apoptosis in the primary cell population as a whole.

FAC binds to a number of cytoplasmic proteins *in vitro* (10) and forms a multimeric complex that may interact with the FA group A protein, FAA, and traffic to the nucleus (12). One of the proteins discovered to bind to the FAC polypeptide is the cyclin-dependent kinase, cdc2 (13). The cdc2 kinase has recently been found to be a critical mediator for Fas-induced apoptosis and protease activation (14), raising the possibility that FAC may influence apoptosis via such an interaction.

IFN-γ and TNF-α can up-regulate Fas receptor expression on cells and thereby induce apoptosis (15). Our results indicate that murine bone marrow cells cultured in SCF, IL-3, and erythropoietin are sensitive to Fas-antibody-triggered cytolysis even without the addition of IFN-γ or TNF-α, consistent with Fas receptor induction by cytokine-supported culture alone (16). The Fas receptor was expressed on approximately 70% of cytokine-stimulated bone marrow cells from transgenic or control animals, as analyzed by flow cytometry.[4] The antiapoptotic effects of FAC are evident in hematopoietic cells triggered directly by an agonist antibody, whereas the effect is less profound with IFN-γ and TNF-α. Possibly, FAC may act in the Fas-mediated apoptosis pathway before the convergence of this pathway with that induced by the combination of IFN-γ and TNF-α (17).

Pathophysiological Model of FA. A knock-out mouse model for FA was recently described (18) in which murine *Fac* was inactivated by targeted mutation. Hematopoietic progenitors from the knock-out mice were found to be hypersensitive to inhibition by IFN-γ (18, 19). In other of our experiments, we demonstrated that hematopoietic progenitors from these *Fac*-null mice were also more sensitive to Fas-triggered cell death than were those from *Fac*-heterozygous mice.[5] Consistent with these two findings, our present work seems to model the reciprocal situation in that the overexpression of human FAC protects against Fas-mediated cell death. By implication, Fanconi anemia—caused by mutation in the *FAC* gene—may be a disorder of apoptosis. In this model, hematopoietic cell death exceeds cell proliferation, leading to stem and progenitor cell depletion and aplastic anemia.

Given our model for FA pathophysiology, one unresolved paradox relates to the tendency of FA patients to develop leukemias. We reported previously (20) that wild-type *FAC* transfection decreased the susceptibility of mutant fibroblasts to transformation by the oncogenic SV40 virus, consistent with a role for wild-type FAC in tumor suppression. If, as we have suggested, wild-type FAC also functions to suppress apoptosis, then defective FAC function manifested by a greater sensitivity to cell death would not account for the tendency of mutant cells to undergo malignant transformation. A possible explanation for these findings may be incomplete cell death of hematopoietic cells that leads to the outgrowth of damaged preleukemic clones. Defining the precise role of *FAC* in these pathways should lead to a better understanding of FA cancer susceptibility as well as of interrelationships among the genes modulating apoptosis.

[4] J. Wang, unpublished observations.

[5] T. Otsuki, S. Nagakura, J. Wang, M. Bloom, and J. M. Liu, submitted for publication.

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