Fanconi Anemia and Novel Strategies for Therapy

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FA CONI ANEMIA (FA), perhaps the best defined of the inherited bone marrow (BM) failure disorders, was first described in three brothers with a syndrome of congenital physical anomalies, anemia, and a fatty aplastic bone marrow. According to Fanconi’s original description, the three boys, all between 5 and 7 years of age, also had microcephaly, intense brown pigmentation of the skin, skin hemorrhages, hypogonadism, internal strabismus, and hyperreflexia. The erythrocytes were hyperchromic and there was no evidence of hemolysis. Subsequently, other FA patients were identified who suffered from leukopenia and thrombocytopenia, in addition to anemia, as a consequence of an aplastic marrow. Malformations of the thumb and kidney were added to the list of physical anomalies. In 1931, Naegeli proposed the name Fanconi’s anemia to distinguish this familial anemia from the exogenous anemias caused by inadequate nutrition.

The modern diagnosis of FA no longer rests on the constellation of abnormalities described by Fanconi but depends on finding chromosomal breakage after incubation of the patient’s cells with chemical clastogens such as diepoxybutane (DEB) or mitomycin C (MMC). This strict laboratory diagnosis has led to the identification of affected individuals but who may be quite heterogeneous in terms of their clinical features. A large number of FA patients may present with congenital malformations but not be diagnosed with FA until the onset of BM failure. The spectrum of congenital malformations has also widened to include abnormalities of the central nervous and gastrointestinal systems. A further problematic aspect of the modern diagnosis of FA is the categorization of certain patients who have some of the physical and hematologic criteria of FA but who have negative chromosome breakage studies. Whether such patients or patients with idiopathic “acquired” aplastic anemia will eventually be found to have mutations in FA or FA-like genes is an open question.

FA usually presents with aplastic anemia, identical to the acquired variety. More recent data compiled from the International Fanconi Anemia Registry (IFAR) suggest that the risk of developing myelodysplastic syndrome or acute myelogenous leukemia may be higher than previously thought. From a scientific perspective, FA may be a model for study of the evolution of preleukemia and acute leukemia as well as aplastic anemia. From a clinical standpoint, FA presents a difficult challenge to the hematologist because FA patients may present with either aplasia or dysplasia. In this review, we suggest a framework for consideration of FA in the context of the cloning and molecular characterization of a FA gene. We conclude with a critical appraisal of the prospects for gene therapy of FA.

FA is usually grouped with inherited cancer-prone syndromes such as ataxia telangiectasia, Bloom’s syndrome, and xeroderma pigmentosum. For some of these disorders, the genetic defect has been identified and correlated with some aspect of DNA repair. For others, such as FA, the relationship to DNA repair is inferential. Adding to the complexity, FA is clearly a heterogeneous disorder with both genetic and phenotypic variability. The likelihood of ascribing all FA defects to a single biochemical DNA repair pathway seems small. In this review, we have attempted to discriminate between general and specific FA defects and have identified the FA complementation group (if known) of cells used in particular experiments (Table 1).

GENERAL FEATURES OF THE FA PHENOTYPE

Sensitivity to DNA cross-linking agents. Susceptibility of FA cells to DNA cross-linking and alkylating agents was first recognized 40 years after the clinical description by Fanconi. Monoadducts and diadducts (interstrand cross-links) are induced in DNA by bifunctional cross-linking agents such as DEB, MMC, nitrogen mustard, cyclophosphamide, cisplatin, or psoralen plus near UV light. Interstrand cross-links are thought to block DNA replication and RNA transcription, with potent effects on cell survival and function. In FA, specific cellular defects after exposure to bifunctional cross-linking agents such as MMC include the induction of chromosomal aberrations (breaks and rearrangements), delayed transit and arrest in the G2 phase of the cell cycle with a consequent decrease in cells synthesizing DNA, and cell death.

G2 phase prolongation and arrest. Cell cycle kinetics in FA have been studied using a specialized flow cytometric technique analyzing cells that are labeled with bromodeoxyuridine (BrdU; incorporated in place of thymidine) and stained with the DNA dyes Hoechst 33258 and ethidium bromide. These types of analyses enable us to make a determination of the fraction of cells that enter but do not exit a particular cell compartment. FA cells accumulate not only in the G2 phase of the first cell cycle, but also within the G2 compartments of the second and third consecutive cell cycles. The phenomenon of G2 phase prolongation and arrest seems to be a general feature of FA cells. At least three other general features have been associated with the FA cellular phenotype: oxygen sensitivity, G2 chromatid radiosensitivity, and tumor necrosis factor (TNF) overproduction. The possible interrelationships between these phenomena are instructive in understanding the nature of the FA defect.

FA cells show abnormal sensitivity to oxygen; they grow...
Table 1. Cell Lines Characterized According to Complementation Group

<table>
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<tr>
<th>Complementation group</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
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<tbody>
<tr>
<td>Lymphoblasts</td>
<td>HSC 72 (GM13022)</td>
<td>HSC230 (GM13071)</td>
<td>HSC536 (GM13020)</td>
<td>HSC62 (GM13023)</td>
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<tr>
<td>HSC 99</td>
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<tr>
<td>HSC 526f</td>
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<tr>
<td>HSC 527f</td>
<td></td>
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<tr>
<td>SV40-transformed fibroblasts</td>
<td>GM 6914t</td>
<td></td>
<td></td>
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<tr>
<td>Primary fibroblasts</td>
<td>156</td>
<td>1199</td>
<td>145</td>
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<tr>
<td></td>
<td>402</td>
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<td></td>
<td>GM1309t</td>
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* HSC 610 and PD-4L (GM12794) are from sisters.
† GM 6914, HSC 526, and HSC 527 are from three brothers. GM6914 was derived by transformation of parental line GM1309.
‡ PD-4L and GM12794 were derived from the same individual.
§ GM13136 was derived by transformation of parental line GM449.

very poorly at ambient (20%) but well at reduced (5%) oxygen tension.16 Elegant BrdU-Hoechst flow cytometric studies have suggested that oxygen sensitivity reflects a tendency of FA fibroblasts to accumulate in the G2 phase of the cell cycle.16 Interference with DNA topoisomerase function may underlie the mechanism of oxygen-exacerbated G2 prolongation.18 Oxygen and its reactive species also can modulate the proliferation of normal diploid fibroblasts, again characteristically inducing G2 delay.19

Oxygen sensitivity in FA may involve either the complex system that controls excessive production of reactive oxygen species or the ability to tolerate oxygen-induced damage. Data supporting an intrinsic hypersensitivity to oxygen, overproduction of reactive oxygen species, as well as a deficient antioxidant defense have been accumulating (Fig 1), but it seems doubtful that these events are causal for the FA defect. Because these topics have been the subject of several recent reviews,24 they will not be detailed here except for two interesting findings. One of the first experiments linking FA with a defect in the enzymatic antioxidant defense system showed that CuZn superoxide dismutase (SOD), a key enzyme that detoxifies the superoxide anion (O$_2^-$), can suppress the cytotoxic effect of MMC on an FA primary fibroblast cell line.25 These results prompted pilot clinical trials of CuZn-SOD that have suggested a beneficial effect on chromosome aberration or cell colony survival.26 On the other hand, FA cells exhibit increased chromatid-type aberrations after irradiation in the G2 phase of the cell cycle.33 In this feature, FA shares similarities with other genetic cancer-predisposing disorders such as ataxia telangiectasia, Gardner's syndrome, Bloom's syndrome, and xeroderma pigmentosum. G2 chromatid radiosensitivity may to the subsequent clastogenic effect of O$_2$ than lymphocytes without MMC pretreatment.31

The effect of ionizing irradiation has been studied in two cell types. In contrast to cells from patients with the genetic disorder ataxia telangiectasia, FA fibroblasts and lymphocytes do not appear to be hypersensitive to ionizing irradiation as measured by chromosome aberration or cell colony survival.32 On the other hand, FA cells exhibit increased chromatid-type aberrations after irradiation in the G2 phase of the cell cycle.33 In this feature, FA shares similarities with other genetic cancer-predisposing disorders such as ataxia telangiectasia, Gardner's syndrome, Bloom's syndrome, and xeroderma pigmentosum. G2 chromatid radiosensitivity may

Fig 1. Schema of the cellular sources of reactive oxygen species and of the antioxidant defense system responsible for detoxifying these species. In the FA erythrocyte (RBC), levels of SOD and glutathione (GSH) are decreased, whereas glutathione (GSH) transferase is increased. In the FA leukocyte (WBC), levels of SOD are also decreased, and reactive oxygen species, including superoxide (O$_2^-$), hydroxyl radical (OH$^-$), and singlet oxygen (O$_2$) may be generated and detected by chemiluminescent assays (CL). In the FA fibroblast, levels of manganese-SOD (MnSOD), catalase (CAT), and glutathione peroxidase (GPx) are paradoxically increased.
result from a defect in G2 repair of DNA damaged by reactive oxygen species.34

Several recent reports have documented overproduction of TNFα from FA lymphoblasts35 and patient serum samples.36 Like oxygen sensitivity, TNFα overproduction seems to be a general feature of the FA phenotype. Up to an eightfold increase in the cytokine was found in the growth medium of FA lymphoblasts.35 Addition of anti-TNFα antibodies partially corrected the FA cellular and chromosomal hypersensitivity to MMC, reminiscent of the experiments with SOD. TNFα is a multifunctional cytokine and an important mediator of oxidative stress.37 TNFα overproduction might be a response to the stress of DNA damage. Eukaryotic cells are known to respond to genotoxic stress via the induction of genes that are involved in the control of cell proliferation and in the repair of DNA.38 TNFα is an example of a pleiotropic gene that is induced in response to DNA damage and, in turn, is able to induce other genes involved in growth control. Conceivably, sensitivity to oxygen (enhanced or exacerbated by TNFα production) may be a reflection of the type of damage that the FA gene recognizes.

We began this analysis of some of the general features of FA by linking the oxygen hypersensitivity of FA cells to cell cycle disturbances. However, although G2 phase prolongation/arrest seems to be a general characteristic of the FA phenotype, it need not be specific for oxygen stress. As noted, MMC is also able to cause a G2 block in FA fibroblasts.12 Arrest of mammalian cells in the G2 phase of the cell cycle occurs after X-irradiation.39 TNFα acts preferentially during the G2 phase and can lead to G2 prolongation.40 It seems plausible that G2 delay and arrest may be triggered by DNA lesions, such as double-strand breaks, generated by the action of oxygen,41 TNFα, or clastogenic agents.42 The G2/M transition is genetically regulated in response to DNA damage and serves as a checkpoint to delay cell cycle progression and to allow for the repair of damaged DNA.43,44 In FA, G2 phase prolongation/arrest seems to be a consequence of DNA damage resulting from defective oxygen metabolism or clastogen-induced breakage rather than a primary cell cycle disturbance. A defective response (recognition or processing) to DNA lesions is likely to be a key feature of FA.

GENETIC ANALYSIS OF FA BY COMPLEMENTATION STUDIES

The extensive clinical and cellular heterogeneity seen in FA has led to the investigation of genetic heterogeneity in this disease, because it could be hypothesized that mutations in different genes could lead to alternative phenotypes. Genetic heterogeneity studies have exploited the increased sensitivity of FA cells to the cytotoxic action of DNA cross-linking agents.14,45-47 FA fibroblast strains were initially classified into two complementation groups based on their ability to correct their chromosomal fragility after fusion.48 However, because these cells could not be propagated easily, it was not possible to study their phenotype in detail. A more extensive study was based on the analysis of cellular properties of hybrid lymphoblast cell lines. Selectable markers were introduced into FA cells and used to isolate hybrids after fusion. Complementation of the FA phenotype was assessed by analysis of spontaneous and MMC-induced chromosomal breakage and of growth inhibition by MMC. The initial studies led to the identification of two complementation groups, A and B (in reality non-A).49 Subsequent analysis of the non-A cell lines has led to the identification of three other complementation groups, ie, B, C, and D.50 Given the small numbers of cell lines examined to date, it is likely that more complementation groups will be identified.

The results of complementation studies lend support to the view that the increased cellular sensitivity to DNA cross-linking agents and the increased spontaneous (and MMC-induced) chromosomal fragility are direct consequences of the gene defect, rather than more distant events that could be strongly influenced by the action of other genes or by environmental effects. In this regard, little analysis has been performed on the other phenotypic manifestations observed in FA cells. The cell cycle defects, increased sensitivity to oxygen toxicity, or putative DNA repair abnormalities have not been analyzed in complementing hybrid cell lines and, thus, their relationship to the basic defect in the disease is not yet known.

POSSIBLE FUNCTION OF FA GENES: DNA DAMAGE

Counteracting the effects of both exogenous and endogenous agents of DNA damage is the function of a complex cellular machinery11 that includes both enzymes and transcription factors as well as regulatory checkpoints that govern entry of the cell into cycle.52 Unraveling these mecha-
nisms has provided important insights into the etiology of genetic instability and malignancy. A key conclusion from these studies is that a defect in DNA repair can be responsible for genetic instability. DNA damage (Fig 2) can include lesions such as pyrimidine dimers, single- and double-stranded breaks, adducts, deletions, base changes, and cross-links that must be recognized and repaired by specific sets of enzymes involved in either direct reversal of damage or excision of the damaged nucleotide or base. In addition to repair processes, the cell has evolved tolerance mechanisms that bypass DNA repair, sometimes allowing the introduction of errors in daughter DNA. Finally, recombination events can also be involved in repair and tolerance responses.

We have already commented on the hypersensitivity of FA cells to oxygen. This sensitivity may reflect a defect in the repair of oxygen radical-damaged DNA. Reactive oxygen species can damage DNA at the base or sugar as well as form complex products such as cross-links and double-strand breaks. Excision repair of a base damaged by oxygen free radicals is mediated by specific DNA glycosylases (Fig 3, right panel). Specific AP endonucleases then break the phosphodiester backbone, resulting in a gap; this gap is subsequently filled and sealed by DNA polymerase and ligase. Assays have been developed that can detect the formation of radical-damaged DNA. For example, 8-hydroxyguanosine (8OH-dG) is derived from deoxyguanosine (dG) by the action of radicals (Fig 4). In two FA(A) lymphoblast cell lines, two to three times more 8OH-dG was formed than in control cells after incubation with H2O2, which is direct evidence of increased susceptibility to oxidative damage of these FA(A) cells. Whether FA can be caused by a defect in an enzyme involved in the repair of oxidatively damaged DNA is unknown.

The sensitivity of FA cells to bifunctional cross-linking agents suggested that the ability to repair DNA cross-links is impaired in FA. Handling of DNA cross-links is thought to be accomplished by the ubiquitous and versatile nucleotide excision pathway (Fig 3, left panel). The steps in this pathway include recognition of damage, incision of the damaged DNA strand, excision of the defective site, repair replication, and ligation to replace the excised region with normal nucleotides. Repair of vertebrate genomes is linked to transcriptional activity; transcribed regions are repaired quickly, whereas untranscribed regions remain unrepaired. Some repair genes actually constitute part of the transcription initiation complex. When RNA polymerase II encounters a DNA lesion, a helicase-like protein known as transcription initiation factor TFIIH may displace the stalled polymerase and unwind the region for entry of DNA repair enzymes and nucleases. Xeroderma pigmentosum (XP) is a well-studied paradigm disorder involving nucleotide excision repair. XP can be caused by mutations in any of at least seven different genes designated A-G. The proteins encoded by two of the XP genes, XPB and XPD, are transient or integral components of TFIIH and apparently shuttle between transcribed and untranscribed regions of the genome.

The fidelity of excision repair has been studied in FA. At first, FA was thought to be comparable to XP in terms of the inability to repair and remove DNA cross-links. However, in contrast to the situation in XP, FA cells apparently are not completely deficient but only exhibit a partial defect in the incision of interstrand cross-links. Two recent reports highlight some of the controversies over the nature of the FA defect in DNA repair. In one study, gene-specific repair was measured for DNA adducts induced by cisplatin in an FA(A) cell line. FA(A) cells were found to repair these interstrand cross-links with only 50% to 60% of the efficiency of normal control cells. In contrast, another group of investigators using similar techniques were unable to differentiate FA cells from normal cells on the basis of comparing MMC-induced cross-link repair efficiency at the level of the ribosomal RNA gene.
interstrand cross-links produced by trimethylpsoralen or 8-methoxypsoralen plus UVA light. In addition, chromatin protein extracts from this cell line were defective for a DNA binding protein with specificity for interstrand cross-links. Thus, the FA repair defect may be due in part to lack of this damage-recognition protein as well as to a defect in the function of an endonuclease complex involved in the DNA incision process.

Postreplication recombination repair and translesion replication represent two alternative pathways to excision repair. Recombinational repair is one of the major mechanisms for handling double-strand breaks as well as DNA cross-links. In this pathway, the DNA polymerase enzyme stops upon encountering a DNA lesion and then resumes replication of DNA downstream; the postreplication gap may be filled via recombination. In bacteria, exposure to agents that interfere with DNA replication (UV damage, mitomycin C, etc) leads to induction of the so-called SOS response. Translesion synthesis is one of the components of the SOS response and refers to non-template-directed or error-prone replication across the DNA lesion, leading to a site of mismatch. Although the biochemistry of these types of repair and bypass have not been characterized well in eukaryotes, these pathways may be useful in conceptualizing the FA phenotype.

One of the concepts central to theories of cancer development is that genomic instability precedes tumorigenesis. An early step in carcinogenesis may be the development of a mutator phenotype in which cells exhibit an increased mutation rate. Hypermutability has recently been linked to a deficiency in mismatch repair in a genetic cancer-predisposing disorder, hereditary nonpolyposis colorectal cancer. An association between hypermutability and other cancer-predisposing disorders might be direct evidence that the fidelity of DNA repair is impaired. Cells from XP patients show higher frequencies of mutations when compared with normal cells, suggesting that unexcised lesions are processed by an intact error-prone mechanism because XP cells lack the error-free excision repair system. In this case, an error-prone pathway might include a mechanism analogous to bacterial translesion synthesis, which leads to mismatch formation.

In FA, the situation is more complex. Although the rate of basepair substitutions was found to be low at the hypoxanthine phosphoribosyl transferase locus in FA(A) and FA(D) lymphoblasts and T lymphocytes, the proportion of deletions was three to six times higher in mutants as compared with normal or XP cells. Furthermore, mutation frequency at the glycoporphin A (GPA) locus was found to be markedly elevated in FA erythrocytes. Hypermutability at the GPA locus again could again be attributed to a predisposition to deletion or chromosome loss as opposed to point mutation. Allelic loss can lead to the dysregulation of cell proliferation by deletion or inactivation of tumor-suppressor genes. In this sense, the chromosome instability of FA may be causally related to predisposition to leukemia and other solid tumors.

**CELL DEATH**

A number of features of the FA phenotype are reminiscent of cellular senescence. Ultrastructural studies of cultured FA fibroblasts have shown gross abnormalities such as multinuclearity and nuclear fragmentation, irregular chromatin distribution, and internuclear chromatin bridges. Micronuclei, known to be induced by clastogenic agents in FA cells, often lacked a nuclear lamina matrix. These changes involving the nucleus mimic those seen in late passage human diploid fibroblasts. FA fibroblasts occasionally exhibited a blunted response to interleukin-1 (IL-1)-induced cytokine gene expression, similar to that seen in stromal cells from elderly individuals. FA fibroblasts lose the ability to repair cross-linked DNA with increased passage number. Finally, FA bears some similarity to another genetic instability disorder, Werner’s syndrome, in DNA ligation assays. Patients with Werner’s syndrome suffer from premature aging of multiple organs as well as a predisposition to malignancy. Plasmids transfected in both FA and Werner’s syndrome lymphoblast lines were prone to higher mutation frequencies after ligation of DNA. These findings support the idea that destabilization and alteration of the genome accompany the cellular changes of senescence.

Cell cycle arrest and the loss of proliferative capacity are hallmarks of both FA and senescent fibroblasts. These cell biologic mechanisms could reflect global defects in cell growth. Mutations in a subset of cells, as a consequence of diminished DNA repair and hypermutability, for example, could enable an escape from senescence, initiating a step in the development of neoplasia. Tumor-suppressor genes such as p53 and the retinoblastoma (RB) gene are intimately involved in halting cell cycle progression, but their role in FA has not been defined. Apoptosis, a morphologically distinct program of cell suicide, may be secondarily triggered by oxidative stress or DNA damage. Mechanisms resembling apoptosis have been implicated in the G2 arrest and cell death induced by bifunctional cross-linking agents such as cisplatin.

**IDENTIFICATION OF THE FA GENES**

*Identification of homologs to FA in other species.* Both to understand the basic biology of FA and as a possible aid to the cloning of FA genes, attempts have been made to identify homologs to FA in other species. Thus, a series of nitrogen mustard (NM)-sensitive strains of Drosophila melanogaster have been studied and characterized. The mus308 mutants were found to have a mitochondrial nuclelease with an altered pH activity profile and this phenotype was subsequently shown to be present in FA(A) fibroblasts and lymphoblasts, but not in other FA complementation groups. Thus, *mus308* is a potential FA(A) homolog and efforts are currently underway to clone the *mus308* gene and to then determine whether the human homolog of this gene is the one defective in FA(A) patients.

An MMC-sensitive Chinese hamster cell line (VH4) has been identified that is not complemented by FA(A) cells but is complemented by those of other complementation groups. Interestingly, VH4 cells also possess the mitochondrial nuclelease with the abnormal pH profile seen in FA(A) cells and *mus308* flies, suggesting that it, too, is a
homolog of FA(A). Attempts are also being made to identify the gene defective in VH4 cells.

**Attempts to identify the FA genes by positional cloning.** If sufficient families are available for the initial assignment of linkage, positional cloning allows the identification of human genes without knowledge of their function. Using the large collection of families obtained through the IFAR, the FA(A) locus was mapped to 20q. Positive linkage scores were obtained only on the assumption of genetic heterogeneity using a computer program that seeks to maximize the linkage score by testing all possible assignments. Although this is a reasonable assumption given the existence of genetic heterogeneity in this disorder, the families used in the linkage analysis had not been classified into complementation groups. Thus, the assignment of families as linked or nonlinked was not based on an independent criterion and the map location must be considered provisional. Further attempts to narrow the location of the FA(A) locus within chromosome 20q have not been successful because of the few families available. It is more likely that the true location of the FA(A) locus will be determined after the cloning of the FA(A) gene by alternative methods.

**Cloning of the FA(C) cDNA by functional complementation.** As an alternative to positional cloning, several groups have attempted to clone the FA genes by exploiting the increased sensitivity of FA cells to DNA cross-linking agents. Recently, cDNA libraries in episomal vectors were successfully used to isolate a series of cDNAs that complement the cellular defects of FA(C) cells. The identity of these cDNAs as the true FA(C) cDNAs was confirmed by showing, first, that the defects in FA(A), (B), or (D) cells were not complemented and, second, by finding a mutation in the cell line used for the cloning. The cDNAs code for a novel protein of approximately 63 kD and have alternative 5' and 3' untranslated sequences. It was postulated that the former represented independent exons and the latter resulted from the use of alternative polyadenylation signals. The success of this approach has encouraged attempts at cloning the other FA genes using similar strategies, but no new genes have been reported to date.

**STRUCTURE AND FUNCTION OF THE FA(C) GENE (FACC)**

**Characteristics of the FA(C) gene and protein.** The FA(C) gene (FACC) has been mapped to 9q22.3 by in situ hybridization. Portions of the gene have been cloned and preliminary studies suggest that FACC is greater than 100 kb in length (Savoia et al, unpublished experiments). The two 5' UTRs are encoded in two exons located approximately 600 bp from each other, that designated exon 1 located 5' to that designated -1a. The 3' UTR is contained in a single exon, confirming that the three different 3' UTRs cloned from the library and observed on Northern blots arise through the use of three different polyadenylation signals rather than from alternative splicing. The coding sequence of the gene is contained in 14 exons, identified by the use of vectorette polymerase chain reaction (PCR) and a yeast artificial chromosome (YAC) containing the gene.

The mouse homolog has also been cloned and, where examined, its coding sequence is also interrupted by introns at the same location (Chen and Buchwald, unpublished observations). The sequence identity between human and mouse cDNAs is 68% and the encoded proteins are identical at 65% of residues and similar at 78% of residues. Thus, more sequence diversity is detected in FACC in comparison to that observed for some of the genes defective in XP. This difference may be caused by differing cellular functions of the XP and FA proteins. XP proteins have been shown to participate in complexes with multiple other proteins, leading to severe constraints in the degree of sequence variation that is permitted. Because the mouse FACC protein complements the cellular defects in human cells, despite the significant sequence divergence, this result might suggest that FACC either does not form complexes with other proteins or that these have fewer structural constraints.

Direct evidence for the function of FACC is still rudimentary. Conflicting results have been published regarding the intracellular location of the protein. In one study, FACC was reported to be both nuclear and cytoplasmic, whereas in another it was shown to be exclusively cytoplasmic. If the defect in FA is in DNA repair, it would be reasonable to expect that, at least part of the time, the protein would be located in the nucleus. If, on the other hand, the defect is in oxygen metabolism, a cytoplasmic location might be predicted. Once the contradictory results of the location of FACC are resolved, it may be possible to choose one or the other hypothesis for more in-depth study of the function of the protein.

**Mutations in patients.** The initial report on the cloning of the FA(C) cDNAs showed that the one transcript present in the patient had a C→T mutation that led to leuss4 being mutated to pro. This type of mutation is deleterious because it changes the secondary structure of the protein. A cDNA in which the mutation was introduced by site-directed mutagenesis produced an inactive protein, suggesting that the C-terminus of the protein was important for its activity. Other mutations have now been detected in FA patients. A splice mutation in intron 4 (IVS4 + 4 A→T) is the predominant mutation in all FA patients of Ashkenazi Jewish origin. Other mutations include δG322, Q13X, R185X, and D195V. Attempts to understand the important residues of the protein by creating alterations using site-directed mutagenesis have confirmed that the C-terminus of the protein is essential for activity. Deletions of amino acids 354-383 and 533-554 lead to inactive proteins, whereas deletions of amino acids 20-46 and 97-111 yield proteins with partial activity. Introduction of epitope tags to the N-terminus of the protein does not alter its activity, suggesting that this portion of the protein is not essential for the activity tested [correction of the MMC and DEB sensitivity of FA(C) cells].

**GENE TRANSFER STUDIES WITH FACC**

**Biologic role of FACC.** FA(C) lymphoblast cell lines from patients bearing different FACC mutations have been successfully complemented using viral vectors. Phen-
The addition of caffeine shortens the duration of G2 phase (top panel), allowing entry of unrepaired DNA into mitosis and increased chromosomal aberrations. Transfer of the FACC cDNA to MMC-treated FA cells also leads to shortening of G2 phase (bottom panel), but chromosomal aberrations are nearly abolished. See text for discussion.

Role of FACC in hematopoiesis. We have shown that CD34-enriched hematopoetic progenitors isolated from FA and FA(C) patients exhibit the same hypersensitivity to MMC characteristic of cultured FA cells. Gene transduction of FA(C) hematopoetic progenitor cells using a viral vector containing the FACC cDNA significantly improved colony formation in methylcellulose culture in the absence, as well as in the presence, of low-dose MMC. Increased colony growth may reflect the genetic rescue of progenitor cells after transduction with a copy of the normal FACC cDNA. FACC mRNA was detected in progenitor cell colonies, suggesting that the growth advantage resulted from the expression of the normal FACC gene. Even in the absence of MMC, transduced CD34+ cells yielded a significantly greater number of viable colonies than mock-infected controls. This self-selecting growth advantage is apparently conferred by FACC. In support of this notion, normal hematopoiesis, as measured by colony growth, is inhibited when the FACC gene is repressed by antisense oligonucleotides incubated with normal BM. Thus, the FACC gene may be involved in the maintenance of hematopoietic cell growth in addition to its role in the cellular response to DNA damage.

HEMATOPOIESIS AND GROWTH FACTORS

In vitro studies of hematopoiesis from FA patients show decreased or absent colony growth. In an early study, hematopoiesis was assessed by in vitro assay of granulocytic (colony-forming unit-culture [CFU-C]) and erythroid (CFU-E) colonies in seven FA children. Numbers of CFU-C and CFU-E were profoundly reduced or absent in narrow cultures from all patients, suggesting that the primary defect was manifested early in hematopoiesis and involved a primitive hematopoietic stem cell, conclusions that were supported by subsequent analyses of myeloid and erythroid colony growth. More recently, erythropoiesis was analyzed in 24 FA patients by standard peripheral blood colony culture. Patients could be grouped into six clinical types, ranging from those with megaloblasts and near-normal erythroid colony growth (groups 1 through 4) to patients with mild (group 5) or no (group 6) cytopenias.

Subtle defects in hematopoietic growth factor production have been documented in FA cells. Hypoproduction of interleukin-6 (IL-6) by FA(A) and FA(D) lymphoblasts and fibroblasts has been demonstrated. Peripheral blood mononuclear cells from FA patients also produce lower amounts of IL-6 after stimulation by phytohemagglutinin. On the other hand, an extensive study of constitutive as well as IL-1-induced expression of hematopoietic growth factor genes showed substantial variability in the induction of IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). As compared with normal controls, responses ranged from blunting to augmentation. Pharmacologic doses of CSFs such as stem cell factor (SCF) can increase in vitro hematopoietic colony growth to some degree. SCF increased the growth of erythroid progenitors in culture by about two-fold. SCF in combination with erythropoietin could increase marrow CFU-E and burst-forming units-erythroid

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(BFU-E) from FA patients, but not to levels seen in normal individuals. The efficacy of SCF in inducing FA colony growth has not been reproduced by other groups.\textsuperscript{128}

Phase I/II clinical trials of hematopoietic CSFs have been conducted to determine if the marrow failure of FA would respond to pharmacologic doses of CSFs.\textsuperscript{129,130} In one study, seven patients (median white blood cell count [WBC] \(2.24 \times 10^9/L\) and median absolute neutrophil count [ANC] \(0.24 \times 10^9/L\)) were entered on a trial of subcutaneous GM-CSF treatment. Most of the patients were dependent on red blood cell (RBC) and platelet transfusions. Therapy consisted of 21 day cycles of GM-CSF at 2.5 to 10 \(\mu g/kg/d\) with a dose escalation if the response was inadequate. The median WBC of responding patients at the end of the study was increased more than threefold to \(7.3 \times 10^9/L\) and the median ANC was increased by more than 12-fold to \(3.05 \times 10^9/L\). One patient became RBC transfusion-free, but no improvement in platelet count was noted in any of the patients. At the end of the study, no patient had developed excess blasts and cytogenetics remained nonclonal in all patients. Two of six patients treated with subcutaneous IL-3\textsuperscript{139} also had an improvement in both median WBC count and ANC, and one patient had decreased RBC and platelet requirements.

Two recent reports have described long-term BM culture (LTBMC) of FA progenitors.\textsuperscript{135,137} In this technique, an autologous stromal layer is established to support growth of hematopoietic cells. Nonadherent cells were harvested from these cultures and assayed for ability to form CFU-GM and to initiate secondary LTBMBC at 35 to 40 days of culture.\textsuperscript{132} An adherent layer could be established from most FA patients' cells. Nonadherent cells increased in number during the first 2 weeks and were mostly differentiated myeloid cells. By days 35 to 40, the adherent layer contained sufficient numbers of cells to initiate secondary LTBMCS as assayed by culture on an irradiated stromal cell line, but the growth of CFU-GM was impaired. These data were taken to indicate that hematopoietic precursor cells able to proliferate and differentiate are present in FA bone marrow.\textsuperscript{132} However, it is important to know whether the number of long-term culture-initiating cells (LTC-IC) is reduced in FA patients compared with that in normal individuals.

What has emerged from studies of LTBMC and growth factor therapy in FA individuals is that hematopoietic progenitor cells capable of proliferation and differentiation are present in the BM of pancytopenic patients. In other words, the FA hematopoietic defect may not be a simple consequence of a limited stem cell pool but may represent a failure of maturation and differentiation. The clinical response of FA patients to growth factors in vivo (as contrasted with the generally poor response of patients with idiopathic or acquired aplastic anemia) further supports this notion. An important implication of this work is that, if successful genetic correction of the FA defect could be achieved, sufficient hematopoietic reserve exists in vivo to make gene therapy feasible.

**ALLOGENEIC BM TRANSPLANTATION (BMT)**

BMT for treatment of the pancytopenia of FA is now well established and has been reviewed.\textsuperscript{133,134} For patients with an unaffected HLA-identical sibling, allogeneic BMT is the treatment of choice once pancytopenia requiring androgen therapy or transfusions develops. Recently, transplantation from alternative donors has been attempted for recipients who lack HLA-identical siblings. Contrary to the experience in disorders such as the primary immunodeficiencies or severe combined immunodeficiency, results with BMT from alternative donors have been poor, with few long-term survivors (R.E. Harris, personal communication, 1994). In patients lacking a fully matched sibling BM donor (the majority of FA individuals), treatment is usually limited to chronic androgen therapy and supportive care with transfusions and treatment of infections.\textsuperscript{1}

**GENE THERAPY**

Genetic correction of hematopoietic progenitor cells from patients bearing FACC mutant genes may soon become possible for the treatment of the hematologic manifestations of this disorder. Gene replacement therapy consists of the insertion of a normal gene into an appropriate target cell to correct for the function of the defective gene. Viral vectors are the primary vehicles for gene transfer because of their inherent efficiency in entering cells and transmitting foreign genetic material. Only recombinant retroviruses and adenovirus-associated virus (AAV) vectors have been shown to stably transduce hematopoietic progenitor cells.\textsuperscript{135} These vectors are both capable of genomic integration into the host cell chromosomal DNA, an important feature if the objective is the permanent restoration of hematopoietic lineages. Hematopoiesis in FA patients is defective, presumably because of a failure in maturation and differentiation of the hematopoietic progenitor pool secondary to DNA damage and cell growth disturbances. Transduction of FA progenitor and stem cells might lead to a sustained production of genetically corrected hematopoietic cells of all lineages. However, transduction of stem cells has been difficult, because of the need for purification and enrichment of a small number of stem cells as well as cell cycling requirements for retroviral transduction.\textsuperscript{136} For FA, gene transduction of hematopoietic cells may circumvent some of these difficulties because of a self-selecting advantage for growth and expansion of gene-corrected hematopoietic cells within the hypoplastic FA BM microenvironment.

Current strategies for gene therapy have focused on the transduction of primitive hematopoietic stem cells. Stem cells can be obtained from a variety of sources, including peripheral blood.\textsuperscript{136} Hematopoietic growth factors can mobilize stem and progenitor cells from the BM into the peripheral blood.\textsuperscript{137} Large numbers of peripheral blood progenitors for gene transduction can be harvested from treated patients with acquired aplastic anemia after the administration of G-CSF.\textsuperscript{138} We have shown that it is possible to harvest as many as \(11 \times 10^9\) mononuclear cells from a pancytopenic FA patient by apheresis after G-CSF administration (J. Liu, unpublished results). The ability of these peripheral blood progenitors to sustain complete lineage reconstitution in an autologous transplantation setting has yet to be demonstrated.

Umbilical cord blood is enriched for hematopoietic stem
and progenitor cells and umbilical cord blood from HLA-matched siblings was first used for hematopoietic reconstitution of several children with FA. Hematopoietic progenitor cells derived from cord blood may be efficiently transduced by viral vectors (Walsh and Liu, unpublished results).

The need for hematopoietic growth factors and chemotherapeutic agents to induce stem cell cycling for retroviral transduction is currently being assessed. Gene marking studies in patients undergoing autologous BMT for malignancies have shown retroviral gene transfer without the use of hematopoietic growth factors. Twenty patients treated with several cycles of chemotherapy for acute myelogenous leukemia and neuroblastoma underwent BM harvesting and retroviral transduction for 6 hours in the absence of cytokines. The efficiency of in vivo gene transfer to progenitor cells ranged from 4% to 6%, the most promising result obtained to date.

In outbred large animal transplantation models, hematopoietic stem cell transduction by retroviral vectors has been disappointingly inefficient, ranging from 0.5% to 5%. These results have prompted efforts to improve viral vector transduction protocols and to explore the use of nonretroviral vectors. AAV vectors may prove to be useful alternatives to standard retroviral vectors. Wild-type AAV, a defective parvovirus, has several attractive features, including lack of pathogenicity in humans and site-specific integration into a locus on chromosome 19. These characteristics may be favorable because tumorigenesis via insertional mutagenesis is a well-described phenomenon with wild-type retroviruses, and the potential for replication-competent retroviruses to cause disease has been established in a nonhuman primate model.

Perhaps the principal concern regarding gene therapy for FA lies in the possibility that transduction of the normal FACC gene might lead to the outgrowth of a leukemic clone. The normal FA gene product, including FACC, is thought to control genomic instability and compelling data exist indicating that the FA phenotype is susceptible to genetic deletions and loss of heterozygosity. We have shown that gene correction with FACC can diminish this genetic instability. If gene therapy can actually correct this phenotype in vivo, it may be possible to directly alter the “preleukemic program” of FA hematopoietic stem cells. Alternatively, expansion of the limited stem cell pool by gene therapy may diminish some of the risk of leukemic clonal evolution. Is there a “point of no return” beyond which transduction with the normal FACC cannot correct genetic instability? It may be that gene therapy is most effective early in the course of FA (patients falling into groups 5 or 6, for example), and one might envision the use of progenitors isolated from banked cord blood. Carefully designed clinical trials should determine the feasibility and utility of gene therapy for FA.

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