Molecular Approaches to the Treatment of Fanconi Anemia: Recent Advances
Tetsuya Otsuki and Johnson M. Liu
Hematology Branch, NHLBI, National Institutes of Health, Bethesda, MD, USA

(Received for publication on November 6, 1997)

Abstract. Fanconi anemia (FA) is an autosomal recessive disorder that leads to aplastic anemia. Cells from FA patients are abnormally sensitive to DNA cross-linking agents such as mitomycin C. FA consists of at least five subgroups (FA-A through -E). The genes defective in the FA-C and FA-A groups have recently been cloned. Transfection of the normal FA gene into mutant cells corrects the hypersensitivity to DNA cross-linking agents and improves cell viability in vitro. The function of the FA gene products is still unclear, however. For patients lacking a compatible bone marrow transplantation donor, an experimental trial of gene therapy for group C FA is ongoing at the National Institutes of Health. (Keio J Med 47 (1): 42–44, March 1998)

Key words: Fanconi anemia, knock-out mouse, interferon-γ, tumor necrosis factor-α, gene therapy

Fanconi anemia (FA) is an autosomal recessive genetic syndrome characterized by progressive bone marrow failure, multiple developmental abnormalities, and an increased incidence of malignancy, especially acute myelocytic leukemia. A characteristic feature of the FA cell is hypersensitivity to DNA cross-linking agents, such as mitomycin C (MMC), diepoxybutane (DEB), cyclophosphamide and cisplatin. In FA cells these agents cause increased cell death, chromosome breakage, and accumulation in the G2 phase of the cell cycle. The modern diagnosis of FA depends upon finding chromosomal breakage after incubation of the patient's cells with MMC or DEB.

FA is a genetically heterogenous disorder and can be divided into at least five groups (FA-A through -E) using somatic cell hybridization and complementation. With analysis of a larger number of FA cell lines, at least eight complementation groups have been discovered thus far. The genes defective in the FA-C and FA-A groups were identified by both expression and, for FA-A, positional cloning methods. Mapping of the gene defective in FA-D to chromosome 3p has been recently accomplished by using microcell-mediated chromosome transfer. As a stem cell disorder, FA can be effectively treated by complete replacement of patient stem cells by those from a histocompatible donor. However, allogeneic stem transplantation (BMT) is currently limited to patients with a matched sibling donor. BMT from alternative donors, while successful in selected cases, is associated with a high risk of graft failure. For FA patients lacking an appropriate donor, we have focused on the development of experimental gene therapy.

In this review we describe recent advances in our understanding of the molecular pathophysiology of FA and the development of FA-C gene therapy.

Molecular Analysis of FA

The first FA gene to be identified was the FAC gene. By expression cloning it was possible to isolate a cDNA that complemented the cellular defects of an FA-C cell line. The 14-exon cDNA encodes a novel protein, termed FAC, of approximately 63 kD and 558 amino acids. The FAC gene has been mapped to chromosome 9q22.3 by in situ hybridization. The length of the genomic DNA is greater than 100 kb.

Approximately 65% of FA patients from Europe and North America belong to complementation group A. The FAA cDNA has recently been identified by employing the same expression cloning strategy as that used to identify FAC. The FAA gene was
mapped to chromosome 16q24.31 and consists of 43 exons (80kb). The molecular weight of the FAA protein is approximately 163 kD.

The function of these FA proteins remains to be clarified. FAA has two overlapping bipartite nuclear localization signals at the N-terminus and is reported to localize primarily to the nucleus. On the other hand, FAC has been shown to localize primarily to the cytoplasm, particularly the perinuclear region. Enforced FAC has been shown to localize primarily to the nucleus. On the other hand, FAC has been shown to localize primarily to the cytoplasm, particularly the perinuclear region. Enforced nuclear expression of FAC by placing a nuclear localization signal at the N-terminus abolished the ability of FAC to complement FA-C cells, suggesting that FAC exerts its function in the cytoplasm. If the defect in FA is in DNA repair, it would be expected that, at least part of the time, the proteins would translocate to the nucleus. It was very recently reported that the FAC and FAA proteins localize predominantly to the cytoplasm, the FAC/FAA complex was found in similar abundance in the cytoplasm and the nucleus. This suggests that FAC and FAA cooperate in some nuclear function such as DNA repair.

An anti-apoptotic function for FAC was recently reported. Overexpression of FAC in factor-dependent hematopoietic progenitor cell lines suppressed apoptosis induced by growth factor withdrawal. FAC gene transfer also suppressed a DNA cross-linker-inducible apoptosis pathway after complementation of FAC mutant lymphoblastoid cell lines. In transgenic mice overexpressing the human FAC protein, hematopoietic progenitors were significantly more resistant to the cytolytic effects of Fas death receptor ligation (Wang et al., unpublished data). These experiments suggest that FAC may function in modulating apoptosis; when FAC is absent or defective, mutant cells may be unusually susceptible to various forms of apoptotic stimuli.

Fac-deficient mice were recently developed via targeted disruption of exon 9 in the murine gene. Surprisingly, no gross hematologic defects were detected in the fac knock-out mice, indicating that a deficiency in Fac alone could not explain all the clinical features of human FA. Although the mice had normal blood cell counts, the colony-forming capacity of bone marrow cells was markedly diminished by the addition of interferon- (IFN-). Abnormally high levels of tumor necrosis factor- (TNF-) have been reported in FA patients. Addition of antibody to TNF- was found to partially correct the cytotoxic hypersensitivity to MMC. Such inhibitory cytokines may be involved in the pathophysiology of FA bone marrow failure. To clarify this point, we are now mating fac knock-out mice and IFN- and/or TNF- transgenic mice. These experiments may better explain the relationship between the function of these FA proteins remains to be clarified. FAA has two overlapping bipartite nuclear localization signals at the N-terminus and is reported to localize primarily to the nucleus. On the other hand, FAC has been shown to localize primarily to the cytoplasm, particularly the perinuclear region. Enforced FAC has been shown to localize primarily to the nucleus. On the other hand, FAC has been shown to localize primarily to the cytoplasm, particularly the perinuclear region. Enforced nuclear expression of FAC by placing a nuclear localization signal at the N-terminus abolished the ability of FAC to complement FA-C cells, suggesting that FAC exerts its function in the cytoplasm. If the defect in FA is in DNA repair, it would be expected that, at least part of the time, the proteins would translocate to the nucleus. It was very recently reported that the FAC and FAA proteins localize predominantly to the cytoplasm, the FAC/FAA complex was found in similar abundance in the cytoplasm and the nucleus. This suggests that FAC and FAA cooperate in some nuclear function such as DNA repair.

An anti-apoptotic function for FAC was recently reported. Overexpression of FAC in factor-dependent hematopoietic progenitor cell lines suppressed apoptosis induced by growth factor withdrawal. FAC gene transfer also suppressed a DNA cross-linker-inducible apoptosis pathway after complementation of FAC mutant lymphoblastoid cell lines. In transgenic mice overexpressing the human FAC protein, hematopoietic progenitors were significantly more resistant to the cytolytic effects of Fas death receptor ligation (Wang et al., unpublished data). These experiments suggest that FAC may function in modulating apoptosis; when FAC is absent or defective, mutant cells may be unusually susceptible to various forms of apoptotic stimuli.

Fac-deficient mice were recently developed via targeted disruption of exon 9 in the murine gene. Surprisingly, no gross hematologic defects were detected in the fac knock-out mice, indicating that a deficiency in Fac alone could not explain all the clinical features of human FA. Although the mice had normal blood cell counts, the colony-forming capacity of bone marrow cells was markedly diminished by the addition of interferon- (IFN-). Abnormally high levels of tumor necrosis factor- (TNF-) have been reported in FA patients. Addition of antibody to TNF- was found to partially correct the cytotoxic hypersensitivity to MMC. Such inhibitory cytokines may be involved in the pathophysiology of FA bone marrow failure. To clarify this point, we are now mating fac knock-out mice and IFN- and/or TNF- transgenic mice. These experiments may better explain the relationship between

bone marrow failure and FAC gene mutation.

**NIH Experimental Trial of FAC Gene Therapy**

An experimental trial of gene therapy for group C FA patients is currently ongoing at the NIH Clinical Center. This trial calls for multiple infusions of transduced peripheral blood progenitor (PBP) cells, based on previous experimental data indicating that retrovirally-marked CD34-enriched PBP cells can contribute to long-term engraftment after autologous transplantation. For each cycle of gene transduction, patients received a 5-day course of granulocyte-colony stimulating factor subcutaneously, followed by 1–3 daily consecutive leukapheresis procedures to collect circulating hematopoietic progenitor cells. After CD34-enrichment, ex vivo transduction was performed with the G1FASvNa.52 retroviral vector (Genetic Therapy Inc.) containing the FAC gene, in the presence of stem cell factor, interleukin-6 (IL-6), and interleukin-3 (IL-3).

Three FA group C patients were entered into our clinical trial, and each patient received 3 or 4 cycles of gene transfer, each consisting of 1 or 2 infusions of the transduced autologous hematopoietic progenitor cells. Following infusion, the FAC transgene was transiently detectable by polymerase-chain reaction (PCR) in peripheral blood cells. Function of the normal FAC transgene was suggested by a marked increase in hematopoietic colonies following successive transduction cycles in all patients. Transient improvement in bone marrow cellularity coincided with this expansion of hematopoietic progenitors. However, despite the in vitro selective advantage resulting from FAC gene transfer, long-term hematopoietic reconstitution with gene-corrected clones was not observed. Follow-up studies showed that the number of PB mononuclear cells marked by the FAC transgene was approximately 1/1000 or fewer, as assessed by PCR methods (unpublished data).

One major problem with gene therapy is the low efficiency of transduction to multipotent hematopoietic stem cells (HSC). If HSC do not replicate often, they may not be susceptible to retroviral transduction. Recently, cytokines have been tested for the ability to expand the growth of HSC in ex vivo culture. A study employing a new stroma-free culture system consisting of a combination of FLT3 ligand and thrombopoietin showed a dramatic expansion in hematopoietic progenitors of all lineages. Such protocols may enable both the expansion and replication of primitive progenitors. It may be possible to combine expansion protocols with FA gene transduction with the aim of targeting larger numbers of cycling HSC.
References