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Hematopoiesis, Oncogenesis, DNA Damage and Repair

Pathogenesis of Bone Marrow Failure and Myelodysplasia in FA

Grover Bagby, MD
Oregon Health Sciences University, Portland

Our laboratory has been attempting to define the mechanism by which bone marrow failure occurs in children with FA. Last year, we published results of our studies that clearly indicate that the protein encoded by the normal group FA-C gene stimulates the growth of bone marrow stem cells. The results were confirmed by the NIH group of Walsh et al in a different way. That group introduced a normal FA-C gene into stem cells of a child with FA of the C group and found that the stem cells grew better *in vitro*.

Our laboratory has now tackled the more complex problem of determining the exact function of the FA-C protein in stem cells. Two general mechanisms were theoretically possible. First, the FA-C protein might function to make signals for growth in a cell more effective. Alternatively, the normal FA-C protein might function to make signals for growth-arrest (known as mitotic inhibition) less effective. By studying the Fanconi anemia mouse cells (created by Grompe's lab) our group has shown that the second mechanism is more likely. We found that the stem cells of the FA mice were much more sensitive to interferon-gamma (a protein known to be a mediator of aplastic anemia) than were the stem cells of the normal (heterozygous) mice. ••

Analysis of the FA-C Protein and its Binding Partners

Alan D'Andrea, MD
Harvard Medical School, Dana-Farber Cancer Institute, Boston

The primary focus of my laboratory is to understand the function of the proteins that are synthesized by the FA genes. There are at least five FA genes, and each gene encodes a different protein. Only the FA-C gene and protein have been isolated at this time. Multiple efforts are underway to identify the other genes and proteins.

We would like to know the cellular role of the FA-C protein. How does the normal presence of this protein prevent the onset of aplastic anemia and cancer? Why does a child with FA, who is deficient in this protein or who expresses an abnormal form of this protein, develop aplastic anemia?

Our laboratory has recently shown that the FA-C protein is found in the cytoplasm of the cell and that it forms a complex with at least two other binding proteins. The identification of these FA-C binding proteins may shed light on the function of this protein complex.

In addition to these studies, we have become interested in the clinical variability of FA. We have found that different FA patients express different abnormal forms of the FA-C protein. Depending on which abnormal forms of the FA-C protein are expressed, patients vary considerably, from those with a comparatively mild form of the disease, few congenital abnormalities, and late onset of anemia, to those with a comparatively severe form of the disease, multiple congenital abnormalities and early onset of anemia. Understanding these differences may allow us to identify critical parts of the FA-C protein required for its function. ••

Metabolism and Intracellular Interactions of FA-C Protein

Hagop Youssoufian, MD
Brigham and Women's Hospital, Boston

We are interested in the fundamental biochemical pathways that are deficient in Fanconi anemia. Our laboratory has focused its attention on the biochemical interactions and function of the FA-C protein. We have identified a group of proteins that bind to FA-C specifically. The cloning and identification of the genes for these proteins may give us further insights about the molecular derangements in Fanconi anemia, as well as about novel strategies for therapeutic interventions. ••

FA-C Protein Characteristics and Isolation of a Possible FA-C-binding Protein

Maureen Hoatlin, PhD
Oregon Health Sciences University, Portland

A defect in any one of at least five genes can lead to FA. One of these genes, called FA-C, has been cloned. The FA-C protein is different in structure from any other protein yet studied, and the function of the protein is unknown. One of the most useful and basic tools scientists use for studying a protein with an unknown function, like the FA-C protein, is an antibody that binds specifically to that protein. A specific antibody can be used in many different assays. For example, it can be used to determine how long the protein is stable, what kinds of cells make the protein, where in the cell the protein is found, how much of the protein is present, and what other proteins are associated with it. Antibodies may also be used in assays to compare characteristics of mutant forms and normal forms of proteins. In collaboration with Grover Bagby's lab, I have made antibodies that specifically recognize the FA-C protein in cells isolated from human blood. One of the advantages of these antibodies is that they are able to detect FA-C protein in a very simple assay called a Western blot. Because this is an easier and faster way to detect the FA-C protein than what has been available, these antibodies will be useful for many biochemical experiments to understand more about Fanconi anemia, including those mentioned above.

Recently, several groups have found that the FA-C protein is required for normal growth and development of bone marrow cells, and that FA-C protein associates with other proteins in bone marrow cells. I am using a new, powerful method, the yeast two-hybrid system, to isolate proteins that interact with FA-C. This method was first developed in 1991, and has already been used successfully to discover new genes that are involved in the function of many different types of proteins. More importantly, the proteins that these new genes encode have been "caught" interacting with other proteins in a cell, so that their function is revealed. I am using several regions of the FA-C protein as a "bait" in this system to fish out interacting proteins from a library of proteins that are expressed in human blood cells. I have isolated several candidate proteins and their genes using the front end and the back end of the FA-C protein as "bait". I am now analyzing each of the genes and proteins that were caught to ensure that they are truly interacting with the FA-C protein. The genes that encode interacting proteins will be sequenced and thoroughly analyzed for biological significance. By studying the interacting proteins, I hope to determine the function of the FA-C protein. Because a defect in any of at least five separate genes can give rise to FA, the FA-C protein may be part of a protein complex or part of a new pathway that controls growth and maturation of blood cells. It is possible that this method will help identify and isolate the genes for the other FA complementation groups, offering the potential for gene therapy for the individuals that have defects in FA genes other than FA-C. ••

Identification of Molecular Therapeutics

Stephen Friend, PhD
Fred Hutchinson Cancer Research Center, Seattle, Washington

There has been a significant fusion of knowledge about rare but important syndromes, such as Fanconi anemia, and a broad base of knowledge about DNA repair and checkpoints in model organisms, such as yeast. In the last five years, people have begun to appreciate how knowledge from these two apparently disparate areas can be brought together to develop new therapeutic strategies. We have recently set up a group in Seattle that is called "The Seattle Project," with funding from the National Cancer Institute and major pharmaceutical companies, to develop new tools for discovering anti-cancer agents. This collaboration between our lab and Lee Hartwell's lab, while not directly applicable to the treatment of FA patients, provides some basic rules and ideas about gaining a deeper understanding of basic biology. ••

We thank these sponsors of the Seventh Annual FA Scientific Symposium November 9–11, 1995:

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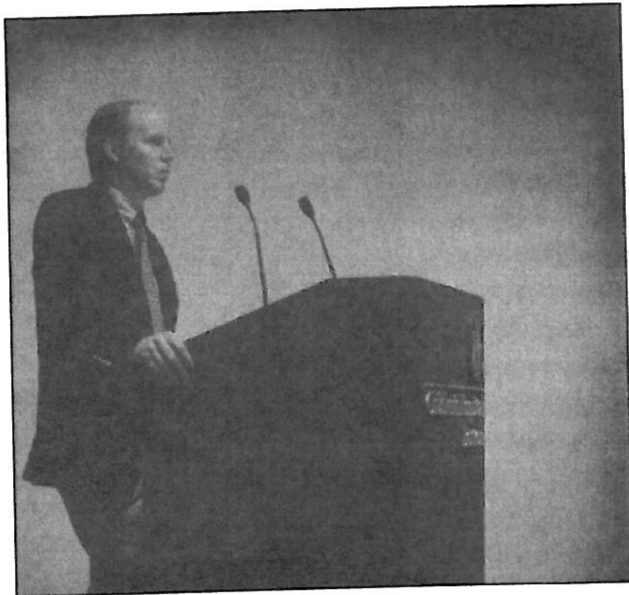
DNA Damage and the Cell Cycle: Analysis of Checkpoint Control Function

Michael Heinrich, MD
Oregon Health Science University, Portland

The process of cell division and replication occurs in an orderly set of steps. The initiation of a step is dependent upon the successful completion of the prior steps. These steps (or compartments) when taken together define the cell cycle.

The cell cycle can be considered to consist of three major compartments: G_1(Growth$_1$), S (Synthesis of DNA), and G_2/M (Growth$_2$/Mitosis). These compartments are analogous to a set of connected rooms in a building. To pass from one room to the next it is necessary to unlock the connecting door. The cell has control mechanisms (locks) that prevent entry into a cell cycle compartment until certain conditions are met. The conditions necessary for passage into the next cell cycle compartment can be thought of as a cellular key.



Cells from individuals with FA are extremely sensitive to compounds that result in crosslinking of DNA. When FA cells are treated with such agents there is an abnormal increase in the percentage of cells that are found in the G_2/M compartment of the cell cycle. Such an accumulation might be caused directly from DNA damage (a defective key) or indirectly due to abnormal cellular control of the cell cycle (a defective lock). Indeed, many FA researchers have speculated that the FA genes may serve to regulate cell cycle control mechanisms.

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A Defective DNA Repair Protein in FA-A

Muriel Lambert, PhD
New Jersey School of Medicine and Dentistry, Newark

FA cells are hypersensitive to the chromosome breaking and cell-killing effects of DNA interstrand crosslinking agents. These crosslinks in DNA inhibit DNA replication and RNA transcription and can lead to loss of cell function and cell death. Cells from individuals with FA have been shown to have a defect in ability to repair damage produced by these agents [reviewed in (1)]. Though the underlying mechanism for this disorder is unknown, it has been hypothesized that a defect in DNA repair may be involved.

In the DNA repair process, one of the most important and critical steps is the initial one in which specific proteins in the cell (damage recognition proteins) recognize the damage, and other proteins (DNA endonucleases) cut the DNA at the site of the damage. In subsequent steps, the damage is removed and the repair process completed. We have isolated a complex of interacting proteins in the nucleus of normal human cells which contains a protein which recognizes the DNA interstrand crosslinks and a DNA endonuclease which incises DNA at these sites of damage (1,2). We have shown that in FA-A cells there is a defect in the ability of this protein complex to recognize and incise DNA containing interstrand crosslinks (1). In particular, FA-A cells are defective in a damage recognition protein which has specificity for DNA interstrand crosslinks (3). The normal complex, when introduced into FA-A cells in culture, can correct the DNA repair defect in these cells. The normal complex can also correct the defect in the ability of the FA-A complex to incise damaged DNA. These results indicate that the normal endonuclease complex contains the protein which, when defective, is responsible for defective DNA repair in FA-A cells.

In our recent studies we have examined the exact nature of the incision defect in repair of DNA interstrand crosslinks in FA-A cells. We have constructed a DNA substrate 132 base pairs in length which contains a single adduct in it. These adducts are either psoralen interstrand crosslinks or psoralen monoadducts, which directly affect only one strand of DNA. Psoralens are a group of naturally occurring compounds found in a variety of common plants which, in conjunction with

continued on page 16 - see Lambert

DNA repair, Oxidative Damage and FA-A Complementation

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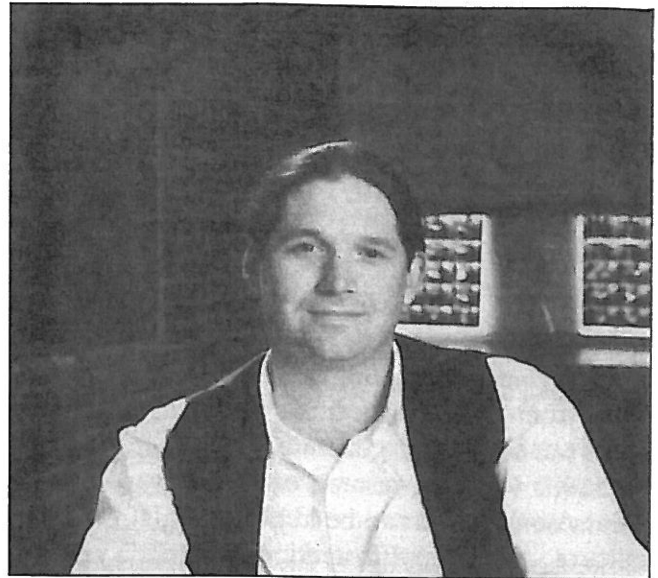


One hypothesis regarding the pathogenesis of FA concerns the hypersensitivity of FA cells to mitomycin C (MMC), a known DNA crosslinker as well as an oxidating DNA damaging agent. The hypothesis is that FA cells are deficient in their ability to repair DNA damage. An alternative hypothesis is that the primary defect is from a faulty oxidative detoxification mechanism, and that the injury to the cell is a consequence of an oxidative injury that results from interactions of the cell with crosslinking agents. A third hypothesis, similar to the second, is that the system that repairs oxygen-induced DNA damage is altered in FA. Therefore, the effects of MMC may be mediated through oxidative DNA damage, as opposed to the DNA crosslinking effects of MMC. Furthermore, previous studies with transformed FA-A lymphoblast cells demonstrated that the FA-A cells have 2 – 3 times more 8-oxoguanine (an abnormal base resulting from oxidative damage) in their DNA than control cells, following incubation with hydrogen peroxide (an oxidizing agent). Obviously, a combination of these hypotheses may be the actual cause of the various phenotypes observed for FA.

The findings presented above for FA MMC-induced DNA crosslinks and oxidative damage led us to investigate the usefulness of a DNA repair gene that acts as an AP lyase (a DNA repair enzyme) and recognizes and removes 8-oxoguanine lesions in DNA. Our previous investigations have shown that ribosomal protein S3 is a multifunctional protein having DNA repair capabilities acting on apurinic/apyrimidinic (AP) sites in DNA and also acting as a combined DNA glycosylase/AP lyase recognizing 8-oxoguanine (7, 8-dihydro-8-oxoguanine) lesions formed in DNA during normal oxidative metabolism. The significance of this DNA repair activity acting on 8-oxoguanine is shown by the ability of S3 to rescue the peroxide sensitivity of an *E. coli* mutM strain that is defective for the repair of 8-oxoguanine, and to abolish completely the mutator phenotype of mutM caused by 8-oxoguanine-mediated G to T transversions. The S3 ribosomal protein is also able to rescue the alkylation sensitivity of an *E. coli* mutant deficient for AP endonuclease activity. Using the *Drosophila* S3 cDNA, we have found that S3 complements the FA-A cells back to wild type levels when challenged with the DNA damaging agent MMC. Therefore, an important component of FA pathology may be the sensitivity of FA-A cells to oxidative damage. We are confirming these findings and performing other experiments with various DNA repair genes that repair oxidative damage in DNA. We are also checking to see whether the other complementation groups are also rescued by the S3 DNA repair gene.

We feel these findings will be of significance in the understanding of the FA-A DNA repair defect, and propose that S3 may eventually be useful in gene therapy for FA-A affected children. ••

Mapping and Cloning of FA Genes

Complementation Studies: an Update

Hans Joenje, PhD
Free University of Amsterdam, The Netherlands

What is complementation?
Cells from two unrelated FA patients can be fused to generate hybrid cells (with 92 instead of 46 chromosomes) that can be kept in culture and examined for mitomycin C (MMC) hypersensitivity as a hallmark of the FA character. Some combinations of cell lines yield hybrids that continue to be MMC hypersensitive, whereas other combinations are less sensitive, like normal cells. These cell lines are said to "complement" (correct) one another. Pairs of unrelated patients whose cell lines yield hybrids that continue to exhibit MMC hypersensitivity (i.e., *do not* complement one another) are said to belong to the same "complementation group." Patients belonging to the same group are presumed to have a defect in the same gene. Based on studies carried out in Buchwald's laboratory, 4 complementation groups were distinguished among 7 unrelated FA patients: groups FA-A, B, C, and D. These 4 groups are thought to represent 4 separate FA genes.

Doing complementation analysis:
The analysis can be carried out only if at least one of the fusion partners is marked with genetic selection markers. Such markers allow selective outgrowth of the (minority of) hybrid cells after the fusion experiment. The (majority of) unwanted

continued on page 19 - see Joenje

Linkage Analysis of FA-A

Fré Arwert, PhD and Chris Mathew, PhD
Free University of Amsterdam, The Netherlands, and Guy's Hospital, London

The work of Buchwald and Joenje has shown that a defect in any of several genes, (FA-A, B, C, D, and E, and possibly F and G), can cause FA. Complementation analysis allows us to tell which gene a patient's defect is in (see Joenje's summary). Most of the FA-C patients can be classified by identifying their specific mutation in the FA-C gene, since this gene has been cloned by Buchwald's group. Liu and Walsh are now investigating whether the FA-C gene can be used in gene therapy. The normal FA-C gene can correct the defect only in group C patients. Group A patients must be treated with the FA-A gene, but so far no one has cloned the FA-A gene. A procedure that has been succesfully used to clone other genes is called "positional cloning." This procedure uses the knowledge of the position of the gene on one of the human chromosomes as a starting point.

In order to find the position of the FA-A gene we have studied FA families that had been assigned to the A group by complementation analysis.Three European labs have collaborated to use DNA from these FA-A families to search for the position of the FA-A gene. Anna Savoia (San Giovanni Rotondo, Italy), Chris Mathew and Rachel Gibson (London, United Kingdom) and Jan Pronk and Fré Arwert (Amsterdam, The Netherlands) collaborating within the framework of EUFAR (European Fanconi Anemia Research) succeeded in finding the location of the FA-A gene by linkage analysis. This procedure makes use of the fact that there are at present at least 600 markers, distributed regularly over all the human chromosomes. The FA-A gene must be linked to one or two of these 600 markers. Genes close together on the same chromosome (in our case, the FA-A gene and one of the 600 markers) tend to be inherited together, or "linked". We investigated which of the markers and the FA-A gene were inherited together in the FA-A families and found that the *FA-A gene was linked to a marker on the tip of the long arm of chromosome 16 (16q24.3)*. This means that we now have determined a region on chromosome 16 where the FA-A gene is located. We have also studied Fanconi anaemia patients from the Afrikaner population of South Africa, which has one of the highest incidences of FA in the world. Most of these patients have been found to be from group A, and about two thirds of them seem to have the same mutation. Genetic typing of these patients has shown that they have only a small region of chromosome 16 in common, and this has allowed us to focus our search for the FA-A gene on a smaller piece of the chromosome. Studies to find and isolate (clone) the gene are in progress, supported by the FA Research Fund, Inc. ••

Complementation by mRNA Microinjection

Martin Digweed, PhD, Humboldt University of Berlin, Germany

Of the many gene transfer techniques available for the identification of sequences which can correct the FA phenotype at the cellular level, we have chosen to concentrate on transfer of messenger RNA (mRNA). The advantage of using mRNA lies in the avoidance of transfer of irrelevant sequences, as in genomic DNA transfer, and the problems of cDNA cloning and expression, as in the case of cDNA-based approaches. Having opted for mRNA transfer, however, there are some constraints imposed on gene identification assays due to the transfer technique itself, microneedle injection, and the transient nature of the cellular response.

The two assays discussed here are (1) DNA replication rate and (2) survival in mitomycin C. FA-A cells show a permanent reduction in DNA replication rates after a crosslinking challenge. In this respect they differ from healthy cells and cells from the other complementation groups. By microinjection of mRNA isolated from human HeLa cells we have been able to correct this abnormal response. Using mRNA fractions of known length confined the correction to mRNAs of approximately 0.7kb, Fraction 21. This fraction contains about 1000 other mRNAs. In an attempt to isolate the correcting sequence we constructed a cDNA library and screened it by microinjection. This approach did not lead to identification of the FA-A gene. However, it was possible to isolate a sequence, SPHAR (S-Phase Response), which has a partial correcting activity and which we have shown to be a cyclin-related gene. Strikingly, control cells, engineered to be deficient in SPHAR and which have reduced DNA replication rates, show two characteristics of FA cells: a prolonged G_2 phase and increased chromosomal breakage after treatment with crosslinking agents. This has led us to hypothesize that the basic defect in FA may be the control of DNA synthesis during S phase. We suggest that incomplete DNA replication may be responsible for spontaneous G_2 phase prolongation and chromosomal damage. Treatment with agents which further disturb DNA replication, such as crosslinkers, necessarily exacerbates the situation.

Isolating the FA-A gene using mRNA Fraction 21 may be possible by following an approach we have demonstrated for the XRCC2 gene which is mutated in irs1 hamster cells. These cells are also highly sensitive to crosslinking agents. By using survival of irs1 cells after a short-term mitomycin C treatment as an assay, we have been able to isolate an mRNA fraction, Fraction 12, containing mRNAs of 3.5 kb which can correct irs1 sensitivity. The XRCC2 gene has been shown by others to be located on human chromosome 7q36.1. By using the Fraction 12 mRNA as a probe, we have identified 30 recombinant cosmids from a chromosome 7-specific library of over 20,000 clones. Most of these cosmids will not stem from the correct sub-chromosomal location. Those that do can be further tested for the XRCC2 gene by transfer into irs1 cells. This powerful combination of a complementing mRNA fraction and positional information is possible for the FA-A gene now that it has been localized. ••

FA-D Complementation Group of Fanconi Anemia

Robb E. Moses, MD
Oregon Health Sciences University, Portland, Oregon

The report from the Moses Laboratory covered three topics with regard to the characteristics of the FA-D complementation group and attempts to identify the gene.

(1) Studies with hydrogen peroxide and methylmethane sulfonate (MMS) showed no difference in toxicity to FA-D (or FA-A) cells compared to normal cells for these two agents. This is interesting because these two agents produce several forms of DNA damage: strand breaks, oxygenated bases, methylated bases and apurinic sites.

The results of the survival tests lead us to conclude that the repair defect in Fanconi anemia, or the changes in cell metabolism, do not include a defect in handling any of these types of DNA damage. In addition, we investigated FA-D and FA-A cells in comparison to normal cells when these two agents were pushed to levels which caused chromosome breaks. Once again, no differences were detected between the FA cells and normal cells with regard to cytogenetic abnormalities. This leads us to conclude that FA cells are capable of repairing single strand breaks as well as normal cells. The conclusion from these studies is that the defect in Fanconi anemia cells reflected in crosslink damage must reflect an early step in DNA crosslink repair. It also raises the possibility that crosslink repair is restricted to the G_2 phase of the cell cycle.

continued on page 16 - see Moses

Linkage Analysis in Fanconi Anemia Families from the International Fanconi Anemia Registry (IFAR)

Arleen D. Auerbach, PhD and Michele Gschwend, PhD
The Rockefeller University, New York, and Stanford University Medical Center, Stanford, California

Auerbach's laboratory has developed amplification refractory mutation system (ARMS) assays for the rapid detection of mutations in the FA-C gene. The results of a study using this assay on DNA samples from over 3,000 healthy Jewish individuals primarily of Ashkenazi descent were recently published in *BLOOD*. We tested these individuals for two of the most common FA-C mutations, IVS4 and 322delG.

Al Gillio, Hal Broxmeyer, and Arleen Auerbach

Our results showed that the carrier frequency of the IVS4 mutation was one in eighty-nine (1.1%); no 322delG carriers were found. This frequency for IVS4 carriers is much higher than was expected and leads us to recommend that screening for the IVS4 mutation merits inclusion in the battery of tests routinely provided to the Jewish population. We also screened over 500 Iraqi Jews in Israel for IVS4, and found no carriers. Since the IVS4 mutation has been found only on chromosomes of Ashkenazi Jewish origin and is the *only* FA-C mutation found on these chromosomes, we hypothesize that a founder effect is responsible for the high frequency of this mutation. In order to test this hypothesis, we have performed linkage analysis with DNA markers tightly linked to the FA-C gene on chromosome 9q22.3. DNA samples from FA patients homozygous for the IVS4 mutation were studied. Markers included in this study were: D9S151, D9S12,

continued on page 17 - see Auerbach and Gschwend

Phenotypic Consequences of Mutations in the FA-C Gene

Alfred Gillio, MD and Arleen D. Auerbach, PhD
Memorial Sloan-Kettering Cancer Center and The Rockefeller University, New York

We present here an analysis of the clinical effects of specific mutations in the FA-C gene. We have developed amplification refractory mutation system (ARMS) assays to detect FA-C mutations rapidly. At least one mutated copy of the FA-C gene was identified in each of 51 FA patients from the International Fanconi Anemia Registry (IFAR). Our analysis suggests that the specific FA-C mutation affects the clinical outcome, and allows division of the patients into three groups: (1) patients with the IVS4 mutation; (2) patients with at least one exon 14 mutation (R548X or L554P); and (3) patients with at least one exon 1 mutation (322delG or Q13X) and no known exon 14 mutation. IVS4 patients are of Ashkenazi Jewish ancestry, while most of the other FA-C patients are of Northern European origin. In this study, the phenotypes of the FA-C patients were compared with each other and also with 338 non-FA-C patients from the IFAR.

Patients with the IVS4 mutation all exhibited a distinct elfin-like facial appearance and multiple congenital malformations. Exon 14 patients also displayed multiple birth defects, while exon 1 patients are usually normal in appearance. Individuals with IVS4 or exon 14 mutations experienced earlier onset of hematologic abnormalities and poorer survival compared to exon 1 patients and to the non-FA-C IFAR population. Four patients in each FA-C group developed acute myelogenous leukemia, for an overall incidence of 24%. The median age at diagnosis of leukemia was younger in the IVS4 and exon 14 groups versus the exon 1 group (8.7 vs. 17.3 years). *The specific mutation in FA-C is thus predictive of the phenotype, with IVS4 and exon 14 being characterized by the presence of multiple birth defects and early onset of life-threatening hematologic abnormalities and leukemia.*

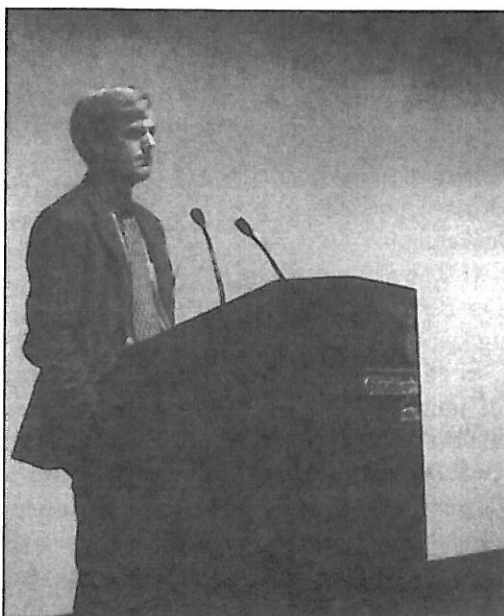
We suggest that FA patients with IVS4 and exon 14 mutations should be followed frequently from birth with blood counts and bone marrow examinations because of the expected early onset of hematologic disease and leukemia. ••

Functional Isolation and Characterization of Stem Cells

David T. Scadden, MD
Massachusetts General Hospital,
Harvard Medical School

The focus of our laboratory is in developing systems to enable us to understand how bone marrow stem cells are regulated. These studies are focused primarily on normal stem cells at present, but may be applied to a number of conditions where stem cell function is altered, including Fanconi anemia.

There are three areas to which our efforts have been directed. The first is in trying to use the known functional properties of bone marrow cells to develop methods of isolating and studying relevant subsets of these cells. Many investigators in this field have demonstrated that the level of maturity of blood cells is reflected by their ability to respond to different growth factors, and that very primitive cells (stem cells), which are capable of regenerating all blood elements, are generally resistant to these growth signals. Cells that *do* respond to these growth signals become sensitive to the effects of certain toxic chemicals which mimic normal nutrients. These so called anti-metabolites may then be used in conjunction with combinations of growth factors to drive most blood cells to a metabolic death, leaving behind only those which are unresponsive to the growth factors. We were able to use this method to purify stem cells quite substantially and were then able to use these cells to investigate some of the molecular properties of the stem cell.



Our second area of interest has been in trying to develop systems whereby we can direct stem cells to undertake maturation along the lines of certain immune cells known as T lymphocytes. The generation of these cells has been difficult to study in humans because systems to evaluate them have generally not been available. Using a combination of certain tissues (the thymus) and specific growth factors, we have now been able to encourage cells from the human bone marrow which have no T cell markers to differentiate into cells that bear T cell markers on their cell surface. In addition, these cells have functional properties of mature T cells. We believe this system will enhance our ability to investigate more fully the potential of the stem cell and expand the tools available to evaluate the regulatory processes acting on the stem cell.

The final area to which we have directed our attention is in trying to find new ways of looking at small numbers of bone marrow cells. Because we are interested in focusing on human diseases and many of the settings in which we can evaluate these diseases force us to deal with very small quantities of cells, there is a need to improve the methods by which we look into the processes governing these cells. A method of gene amplification from single cells has been developed by others. We modified this method to make it a quantitative tool to permit more precise analysis of the levels of gene expression within bone marrow cells. In addition, we combined this with a technique of micromanipulation which allowed us to isolate single cells, grow them under specific conditions and sequentially isolate individual daughter cells dividing from them. By coupling these two methods, we have been able to plot how levels of gene expression change in accompaniment with differentiation of stem cells. This provides a dynamic portrayal of the molecular events occurring within cells as they mature. We believe these methods will provide insight into the temporal relationship of specific events within normal developing blood cells that may prove valuable in understanding disease states such as Fanconi anemia. ••

Update of FA Transplant Registry Data

Dr. Richard E. Harris, MD
Children's Hospital Medical Center, Cincinnati, Ohio

This is an update of marrow transplantation from the Fanconi Anemia Transplant Registry (FATR), the International Bone Marrow Transplant Registry (IBMTR), and from our own transplant center in Cincinnati.

Currently there is information on 390 patients with FA who have undergone marrow transplant in the database of the FATR. This includes 267 matched sibling donor (MSD) transplants, 52 related donor transplants other than from MSDs (Haplo), and 71 unrelated donor transplants (URD). Just over half (51%) of the patients were under the age of 10 years at the time of the transplant; the median age was 9.8 years and the range was from 6 months to 39 years. The median age was 9.8 years for those undergoing MSD transplant , 8.5 years for Haplo, and 11.6 years for URD. Fifty-three percent were males.

The overall survival for the 267 MSD recipients is 60% at 4 years after BMT. Outcome has improved over the years: survival at 4 years for those transplanted from 1990 to the present is 68%; from 1985 - 89 is 57%; and before 1985 is 42%. The survival for children transplanted before age 10 years is 72% at 4 years compared to 48% for those over 10 years of age at time of transplant. Those who were on androgen therapy prior to transplant have a 4 year survival of 66% compared to 90% for those who never received androgens. Those transplanted prior to the development of a cytogenetic clone, myelodysplasia, or leukemia is 72% at 5 years after BMT vs 39% for those with any of these adverse factors. Survival at 4 years after BMT for those who received low dose Cytoxan (<30 mg/kg) as part of the transplant preparative therapy is 70% vs 45% for those receiving doses over 30 mg/kg. A dose of limited field irradiation above or below 450 cGy did not make a difference in outcome (62% vs 67% at 4 years, p=0.78). Likewise, antithymocyte globulin (ATG) did not significantly influence outcome (64% vs 59% at 4 years, p=0.28). The number of transfusions prior to transplant *did* fluence outcome. Previous data from the IBMTR demonstrates that the best survival is in those patients who received a combination of low dose thoraco-abdominal irradiation (<550 cGy) in combination with low dose Cytoxan (<30 mg/kg). The worst outcome is in those patients who received over 100 mg/kg of Cytoxan. This result has also been confirmed in the FATR. The overall incidence of grade II to IV graft vs host disease (GVHD) in the FATR patients was 27%, and of grade III to IV GVHD was 11%. Chronic GVHD was seen in 33% of patients surviving beyond day +100.

Results of 21 MSD transplants at Cincinnati: All patients received Cytoxan 20 mg/kg, thoraco-abdominal irradiation 400 cGy, ATG pre and post BMT, and cyclosporin for graft rejection and graft vs host disease prophylaxis. Nineteen (91%) of the patients are alive and fully engrafted. None developed grade II or greater GVHD. The only two deaths were from relapse of chronic myelomonocytic leukemia in one patient and hemolytic uremic syndrome in the other (an unusual complication after transplant).

Alternative transplants include family members other than a matched sibling, unrelated donors, matched sibling cord blood transplants, and unrelated cord blood transplants. Results of 44 related donor cord blood transplants were recently reported (*Lancet* 346: 214-219, 1995). The potential advantages of cord blood transplants over marrow transplants include a lower incidence of GVHD, a lower risk to the donor, and no increased risk of graft rejection. The first cord blood transplant was performed in Paris in a patient with FA in 1988. I performed the first cord blood transplant in the USA at Cincinnati in 1989, also in a patient with FA. In the *Lancet* article, the incidence of acute and chronic GVHD was only 3% and 6% respectively. Engraftment was documented in 82% of patients and survival was 72% overall (78% in those transplanted for non-malignant conditions such as FA).

Potential complications of unrelated donor BMT include more severe GVHD, graft rejection and infectious complications as well as organ toxicities due to the higher doses of preparative therapy given to the patients to ensure engraftment. In the FATR, the survival at 4 years for unrelated and related unmatched transplants for FA is only 29% and 28% respectively, compared to 60% for MSD transplants. Survival was higher when a fully matched unrelated or related donor was utilized compared to only partially matched donors.

In summary, the best transplant outcome is with matched sibling donors when the transplant is done prior to age 10, prior to transfusions or androgen therapy, and prior to the development of a clone, myelodysplasia, or leukemia. The best survival is seen with regimens which include low dose Cytoxan combined with low dose limited field irradiation. Patients without a matched sibling donor can often be offered a transplant from some other relative or from an unrelated donor, but outcomes are not as good as those seen with MSDs. Future approaches include the use of unrelated cord blood transplants and T-cell depleted unrelated volunteer donor transplants. Several institutions have joined together, including Cincinnati, Minnesota, Sloan-Kettering, and several others in the USA and Europe, to study these two approaches with a common transplant preparative regimen consisting of Cytoxan 40 mg/kg, total body irradiation 450 cGy, and ATG pre-BMT in addition to cyclosporin post BMT. The unrelated volunteer donor marrows will be T-cell depleted, but not the unrelated cord blood units. Preliminary results with this approach appear very encouraging. See Dr. John Wagner's article on page 11. ••

Transplantation of Unrelated Donor Hematopoietic Stem Cells in Patients with FA

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Introduction.
Patients with FA commonly develop bone marrow failure which may evolve into either myelodysplasia or acute myeloid leukemia (AML). Treatment of patients with FA is complicated by their marked hypersensitivity to DNA crosslinking agents, making them particularly sensitive to specific drugs, like Cytoxan (cyclophosphamide) and irradiation. Early experience with allogeneic bone marrow transplantation (BMT) reveals higher than expected side effects as compared to non-Fanconi patients.

Bone marrow transplant results are excellent in young patients with FA transplanted with marrow from HLA-identical sibling donors. Unfortunately, the majority of patients do not have suitably matched sibling donors, making it necessary to investigate the option of mismatched related donor and unrelated donor transplantation. Transplant centers worldwide have recently started investigating the feasibility of using marrow or umbilical cord blood from unrelated donors. The results are summarized below.

Unrelated Donor Bone Marrow Transplantation.
Of 41 alternate donor transplants for FA reported to the *International Bone Marrow Transplant Registry*, the actuarial probability of survival at 2 years was 34%. However, the majority of patients evaluated in this analysis had related donors other than HLA-identical siblings; only 19 patients had unrelated donors. The small number of patients with unrelated donors prevented any analysis within this group alone.

Clinical Studies at the University of Minnesota.
Over the past decade, experience with unrelated donor BMT has grown remarkably. Approximately 400 transplants using unrelated marrow or umbilical cord blood have been performed at the University of Minnesota alone. Dr. Stella Davies, director of the unrelated transplant program, recently analyzed the outcomes of the first 211 unrelated donor transplants at the University of Minnesota between May 1985 and December 1992. Patients were treated for various hematologic disorders (e.g., leukemia, aplastic anemia, immune deficiency disorders); donors were HLA-matched or one antigen mismatched with the patient; and all patients received *unmanipulated* marrow. The analysis revealed that (1) older patient age and (2) transplant from an unrelated donor with a major HLA-A or B mismatch were factors predicting a poor chance of survival in the group of patients as a whole. While overall survival decreased for each decade (patient age), further analysis revealed HLA mismatch at HLA-A or B did not affect survival for patients under 18 years of age. Survival at 3 years after transplant was 53% for patients receiving matched marrow and 41% for patients receiving 1 antigen mismatched marrow (these results are not statistically different). Importantly, the overall incidence of acute graft vs host disease (GVHD) was 56% for children and 72% for adults. There was no difference in risk of GVHD between those receiving a matched versus 1 antigen mismatched unrelated BMT in this analysis.

Between 1990 and 1994, 7 patients with FA were treated by unrelated donor BMT at the University of Minnesota (2 with bone marrow failure only and transfusion dependent; 2 with myelodysplastic syndrome without leukemia; and 3 with frank leukemia with 5-25% blasts in the marrow at the time of BMT). Patients were first treated with Cytoxan 5-10 mg/kg/day for 4 days and total body irradiation 400-450 cGy once. GVHD preventative therapy consisted of cyclosporin and methotrexate in 5 patients and methotrexate, prednisone and XomaZyme in 2. Two received HLA-matched marrow and 5 received HLA mismatched marrow. The marrow was infused *without* T cell depletion. Of the 7 patients, 5 could be evaluated for engraftment and GVHD. Four of the 5 patients engrafted; one had transient recovery of white cells and then lost the graft. Three patients had mild to moderate GVHD and 2 had lethal grade IV disease. Of the 7 patients, 3 are alive 1 to 3.5 years after transplant.

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Cord Blood for Gene Transfer and Transplantation

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Blood derived from the human umbilical cord and placenta at the birth of a child was not too long ago considered a discard material. Once it was demonstrated to contain hematopoietic stem and progenitor cells, those cells necessary for producing the circulating blood elements, and that it could be successfully frozen (cryopreserved), cord blood was used for transplantation. Since the first cord blood transplant for Fanconi anemia in 1988 and for leukemia in 1990, there have been more than 155 transplants performed using frozen cord blood cells either from a sibling (> 65) or from an unrelated donor (> 90). Engraftment rates are high, and graft vs host disease relatively low, with cord blood cells. The first recipient of a cord blood transplant is now 7 years past transplant and cured of the hematological manifestations of the disease. Accumulating evidence now suggests that single collections of cord blood can be used to engraft not only small-sized children, but also large children and adults, ranging in weight up to 80 kg.

Now that cord blood has been shown to contain long-term marrow engrafting stem cells which have therapeutic benefit for a large number of malignant and non-malignant disorders, it is also being seriously considered for potential use in a gene therapy setting. To this end, both retroviral and adeno-associated virus (AAV) vectors, containing a gene of choice, are being studied to place new genetic material into defective cells, such as those from patients with FA-C. Cord blood stem and progenitor cells appear to be good candidate cellular vehicles for gene transfer based on their high proliferative capacity and the ease of transduction of new genes into these cells. Most information in this area is derived from retroviral vectors. The first clinical trials in this area used retroviral vectors containing the adenosine deaminase (ADA) gene to place this gene into cord blood cells from patients with ADA-deficient severe combined immunodeficiency. While it is clear that these ADA-gene transduced cord blood cells have grown in the autologous recipient (the person from whom the cells were derived), it is too early to know if this maneuver has resulted in actual therapy. In the meantime, studies are underway to determine the effects of placing genes for growth factors or growth factor receptors into cells to enhance the proliferative capacity of cells from patients with aplasia. In this context, AAV vectors are also being studied. While less is known about AAV compared to retroviral vectors, AAV vectors have been used to place functional new genetic material into cells. It is clear that we have learned much in a short time regarding use of cord blood as a source of transplantable cells, and as potential cellular vehicles to carry new and non-mutant genetic material into cells. But there is still much research required to determine the best means to utilize these new technologies for optimal therapeutic benefit. ••

Development of Animal Models for FA

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The objective of this research is to develop mouse models for FA-C. Such models can serve various useful roles. First, they may help us understand the relationship between the various alterations in the defective FA-C gene and the clinical picture seen in patients. This type of study cannot be done using cultured cells. Second, mouse models can be used to define the other genetic and environmental factors modifying the defect in FA patients, since we know that there is considerable variability among patients. This likely reflects the modulating effect of other genes (since we are all, except for identical twins, genetically different) and/or environmental influences such as the intrauterine environment, inadvertent exposure to drugs, etc. Finally, mouse models can be helpful for testing novel treatments. In all of this it is important to remember that when we talk of mouse models, we really mean "models". Mice are not humans and there will very likely be differences between mice that are defective in FA-C and human patients. These will reflect fundamental biological differences between the two species. We must then understand what these are and what they tell us about the role FA-C protein plays in biology.

Where is the FA-C gene expressed during mouse development?

When we talk about expression in this context, we mean that the gene is active. Not surprisingly, we found

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Role of the FA-C Gene in Apoptosis

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Fanconi anemia is a disorder characterized by bone marrow failure, increased sensitivity to DNA-crosslinking agents, and propensity for cancer. Programmed cell death (apoptosis) is a natural mechanism for orderly cell death and replacement. In the absence of apoptosis, the cells may undergo abnormal transformation, leading to cancer. We examined the potential role of the FA-C gene in apoptosis in FA-C mutant lymphoblastoid cells before and after stable correction with the normal FA-C gene using the particle-bombardment method (ACCELL® Gene Gun). Camptothecin (Cpt) and β-lapachone (β-lap), both topoisomerase I inhibitors, are known to induce apoptosis in a number of human cells. We evaluated the extent of apoptosis following exposure to Cpt and β-lap in normal human (NL), FA-C (PD149L), and stably-corrected FA-C lymphoblastoid cells (PD149L+FAC). In NL cells, exposure to Cpt or β-lap resulted in 16±2% and 28±7% apoptosis above baseline, respectively; whereas in PD149L cells, exposure to Cpt and β-lap resulted in only 10±2% and 10±3% apoptosis, respectively. However, the stably-corrected cells (PD149L+FAC) exposed to Cpt and β-lap exhibited 35±1% and 25±13% apoptosis, respectively. Our data indicate that the FA-C gene corrects the decreased response to these two apoptosis-inducing agents. ••

The FA-C Δexon9 Knock-out Mouse

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Animal models of human disease can be very valuable in understanding the basic defects of a condition and developing new and improved therapy, including gene therapy. In order to generate a model for Fanconi anemia type C, we used gene knock-out technology in mice. An important piece of the FA-C gene (exon 9) was removed in mouse embryos by genetic engineering, and the animals were allowed to develop to term. Mice carrying the FA-C mutation (FACKO for FA-C knock-out) were bred, and we now have established a colony of such animals, consisting of normal mice (wild-type), mutation carriers (heterozygotes) and affecteds (homozygous mutants).

Initially, we could not detect any differences between FA-C mutant mice and their litter mates. They have normal weight, do not have limb abnormalities and do not have any obvious blood count abnormalities. However, when we studied cultured cells derived from the mice, we saw chromosome breakage identical to what is seen in human FA. This indicated that these animals really had the same defect as human patients.

When the mice became old enough to breed (older than 2 months) a problem became obvious; female FACKO mice were infertile. At first FACKO males appeared to have normal fertility, but we now know that they become less fertile with age. The reason for the reduced fertility became obvious when the ovaries and testes of mutant animals were studied. FACKO ovaries are only 1/10 the size of normal ovaries and have very few eggs (germ cells) in them. Similarly, the testes of affected males are small and contain areas that are totally devoid of sperm formation.

Next, we collaborated with Grover Bagby's group in the analysis of bone marrow cells from FACKO mice. He made a very interesting discovery. While the total number of colonies formed by FACKO marrow is not different from normal mice at 2 months of age, the mutant cells are very sensitive to γ-interferon (IFG). γ-interferon is a substance made by the body in response to infection and stress. It appears from our studies that IFG is more toxic to FA bone marrow than to normal marrow. This observation suggests that the progressive anemia in FA may be due to loss of bone marrow stem cells in response to IFG and similar substances. The idea that blood-forming stem cells are more susceptible to cell death-inducing substances made in the body would also fit with the progressive loss of sperm and egg progenitors, which are another kind of stem cell. It is too early to tell whether this really is the case, but the FACKO mouse affords us the ability to study this process in detail and to test drugs which might oppose the effects of IFG. ••

Cationic Liposome-based *in vivo* Gene Transfer and Expression

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California Pacific Medical Research Institute

We are using cationic liposome-based *in vivo* gene transfer both to create somatic cell transgenic animals and to develop effective gene therapies. We are characterizing the level, duration and cellular-specificity of gene expression produced by cationic liposome-mediated gene delivery in animals. We have found that by selectively altering 4 parameters: (1) cationic liposome formulation, (2) expression vector construction, (3) formation of the DNA:liposome complex and (4) the host milieu, we can increase the efficiency of *in vivo* gene transfer and expression by up to six orders of magnitude, following intravenous injection of DNA:liposome complexes into mice. We have now produced efficient, *in vivo* gene transfer and expression of a variety of genes in various tissues following intraperitoneal, aerosol, spinal cord, or lateral ventricle (brain) administration of DNA:liposome complexes into adult animals, as well as in fetuses, by injecting these complexes directly into fetuses in pregnant rats. The duration of gene expression at some sites can be prolonged.

More recently, we have shown that the mouse GM-CSF and human G-CSF genes can be safely expressed at high levels in animals using this approach. Cationic liposome-mediated delivery of the GM-CSF gene can produce significant anti-bacterial and anti-inflammatory activity in rats challenged intratracheally with a virulent strain of *Pseudomonas aeruginosa* bacteria. Cationic liposome-mediated gene delivery does not cause significant host inflammatory or immune responses. We have shown that repeated intravenous injection of DNA:liposome complexes continues to produce high level systemic transfection upon re-injection. Thus, unlike the case for recombinant adenoviral vectors, a neutralizing host immune response does not limit re-expression, following re-injection of DNA:liposome complexes. Currently, we are attempting to define better the host factors which determine the efficiency and sites of liposome-mediated gene transfer *in vivo*. ••

Parvovirus-based Vectors for Human Gene Therapy

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Our laboratory has had a long-term interest in a human virus called the adeno-associated virus (AAV), which causes no known disease in humans. Approximately 90% of the human population has been infected by this virus. This unique property of AAV has generated a significant interest in its use as a vector for human gene therapy. Current efforts in our laboratory have focused on studying the details of AAV interaction with primary human bone marrow cells, tissue-specific expression of transduced genes, and AAV-mediated transduction and expression in a mouse model to evaluate further the safety and the efficacy of this vector system. Our studies have revealed that AAV infection of human bone marrow cells requires a specific cell surface receptor. In order to further develop AAV-based vectors that mediate stable transduction of primary human bone marrow cells, and also lead to tissue-specific expression, we have incorporated the remarkable tissue-tropism of yet another human virus, parvovirus B19. We constructed an AAV-B19 hybrid vector, and documented that tissue-specific expression of a number of reporter genes and the normal human globin gene does indeed occur in primary human bone marrow cells. We have also evaluated the safety and the efficacy of the AAV-mediated transduction in laboratory mice. These studies were facilitated by our ability to generate purified, high concentrations (~10^{12} particles/ml) of recombinant AAV vectors.

Details of three sets of studies:
First, high-titer, purified vectors were injected directly into the tail-vein of mice (~10^{10} particles/animal) in order to follow the fate of the injected vectors. Second, bone marrow cells from mice were infected with the recombinant AAV containing a reporter gene, transplanted into lethally-irradiated recipient mice, and analyzed for transduction and short-term expression of the transduced gene in the spleen 12-days post-transplantation. And third, bone marrow cells infected with the recombinant AAV–globin virus were used to examine the potential of transduction and long-term expression of the transduced human globin gene 4-months post-transplantation.

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Gene Therapy for Fanconi Anemia

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Replacement of a normal copy of the FA-C gene in the blood-forming (hematopoietic) cells which originate in the bone marrow may improve the hematologic complications of FA-C patients. We have shown in the laboratory that transfer of the FA-C gene will improve the growth of early bone marrow progenitor cells. This has prompted research to understand and improve the transfer of genes into human blood-forming cells. Viruses appear to be the most efficient means of transferring genes into cells. Retroviral vectors have been extensively studied and most frequently used in clinical gene therapy trials. A clinical protocol using a retroviral-based vector carrying the FA-C gene is ongoing at NIH. Adeno-associated virus vectors (AAV) serve as an alternative vector system and have several potential advantages. This virus is normally non-pathogenic (does not produce disease), it can be purified easily, can infect a very broad range of different cell types (including hematopoietic cells), it does not require that cells be actively dividing to infect them (unlike retroviruses), and it can integrate into the target cell chromosomes. Work in our laboratory suggests that this vector may be useful for the treatment of FA. We have demonstrated that this vector carrying the FA-C gene can also infect early hematopoietic progenitor cells in the laboratory, as well as in mice, and more recently, primates. Work is currently focused on developing strategies to improve gene transfer for clinical trials.••

FA-C Gene Therapy Trial

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FA is a genetic disorder characterized by progressive marrow failure and diagnosed by sensitivity to DNA crosslinking agents such as mitomycin C (MMC). Previously, we described the use of viral vectors expressing the normal FA-C gene to correct the phenotype of FA-C mutant blood-forming cells. Gene-corrected cells exhibited markedly improved colony growth in the absence and presence of MMC, suggesting that *ex vivo* transduction followed by autologous (self) cell reinfusion had potential as therapy. We also demonstrated transduction of the normal FA-C gene to bone marrow cells repeatedly infused and transplanted to hematopoietic (blood-forming) tissues of non-myeloablated (not treated to destroy their marrow) mice.

Our clinical trial, based on these findings, tests two questions: (1) Given the inefficiency of retroviral vectors in transducing human hematopoietic stem cells, can that efficiency be improved by multiple cycles of gene therapy in a non-myeloablated host? (2) Can FA-C gene-complemented stem and progenitor cells outcompete defective mutant cells *in vivo* and selectively repopulate patient bone marrow?

The first patient treated was a 10 year-old boy with an FA-C 322delG mutation. We had previously documented that FA-C gene transduction could improve the colony/cluster growth of hematopoietic progenitors from this child. Optimal conditions for gene transfer required vector supernatant and recombinant growth factors. The patient's baseline blood counts included a WBC of 2800/mm^3 (ANC 532/mm^3), Hgb of 8.2 g/dl, and a platelet count of 25,000/mm^3. He had no compatible donors for marrow transplantation and had been treated with oxymetholone since age 8. There were no clonal abnormalities in the marrow. After G-CSF mobilization, he underwent 3 10-liter apheresis procedures yielding a total of 3 x 10^{10} mononuclear and 12 x 10^6 CD34$^+$ cells. Following positive selection and a 72 hour supernatant transduction protocol, 2 x 10^6 CD34$^+$ cells were reinfused without prior myeloablation. Approximately 50% of CFU-C were marked by the FA-C vector, and transduction led to a two-fold improvement in colony formation in the presence of 5nM MMC. Following reinfusion of transduced CD34$^+$ cells, the patient has done well clinically.

Johnson Liu and Christopher Walsh

On continued treatment with G-CSF and oxymetholone, he has a WBC of 17,800/mm^3 (ANC 14,200/mm^3), Hgb of 11.9 g/dl, and a platelet count of 40,000/mm^3. PCR analysis of peripheral blood mononuclear cells (PBMNC) at 1, 2, and 3 months following reinfusion revealed FA-C proviral marking of 0.001-0.01% of PBMNC (granulocytes, T and B lymphocytes). Serial determinations of PBMNC gene transduction as well as MMC cytotoxicity assays should enable us to test the premise of competitive growth advantage and therapeutic potential of reconstituting autologous gene-corrected hematopoietic cells. ••

Heinrich - continued from page 4

In our work we have sought to test systematically whether the cell cycle abnormalities in FA-C cells are secondary to DNA damage, or represent a primary defect in cell cycle control. Introduction of the normal FA-C gene into FA-C lymphoblasts results in a normal cell cycle response to crosslinking agents. However, this result does not distinguish between a defective key or defective lock mechanism because these cells have less DNA damage than uncorrected FA-C cells at any given dose of crosslinking agent.

To test cell cycle control mechanisms directly in FA-C cells, we used different doses of crosslinking agents in normal cells and FA-C cells so as to generate the same amount of DNA damage. When normal cells have the same amount of DNA damage as FA-C cells, they exhibit the same cell cycle abnormalities. Therefore, the cell cycle abnormalities in FA-C cells represent an appropriate response to DNA damage (defective key) and the FA-C protein does not function directly to regulate cell cycle control (normal lock). Using other agents (hydroxyurea and radiation) that alter cell cycle function we found no evidence of any defective cell cycle control mechanisms in FA-C cells. We are currently performing experiments to confirm our results in FA cells from non-C complementation groups and in cells derived from Grompe's mouse model of FA-C. ••

Check Jeff Janock's FA Web Page:
http://bc.cybernex.net/~jj/fa

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(2) Our studies to identify the FA-D gene have produced candidate genes recovered from the cDNA expression library. However, to date, none of the recovered cDNAs represent the authentic FA-D gene. These studies are continuing.

(3) The localization of the FA-D gene has moved forward significantly under the direction of Markus Grompe and Matt Thayer. Their two laboratory groups, using microcell transfer, have identified chromosome 3 as carrying the FA-D gene. Further studies with deletion constructs in chromosome 3 have shown that *the FA-D gene resides in 3p in the 22-24 region.* This represents a significant step forward in identifying the location of the FA-D gene. ••

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long wavelength (UVA) light, are one of the most definitive agents for producing interstrand crosslinks and monoadducts in DNA. We have shown by sequence analysis that the normal protein complexes contain DNA endonucleases that produce incisions in DNA on both sides of psoralen monoadducts and interstrand crosslinks (4). In FA-A cells, however, there is a defect in ability to produce dual incisions endonucleolytically at the site of a crosslink. An incision is produced on only one side of the adduct. Whether the defect is in the DNA endonuclease itself or in the damage recognition protein, which may aid in the recruitment of the endonuclease to the site of damage, is under investigation.

As an aid in the isolation of the proteins involved in repair of DNA interstrand crosslinks, we have developed monoclonal antibodies (Mabs) against proteins in the normal endonuclease complex which recognizes this type of damage. Functional analysis using these Mabs has allowed us to identify a protein which is involved in the initial steps in the DNA repair process and which is defective in FA-A cells. Whether this protein is the defective damage recognition protein or not is currently under investigation, as is the isolation of this protein. Once we have obtained the protein, it will be possible to clone the gene which encodes it and to determine its precise role in the DNA repair process.

The question remains as to how this repair defect in FA-A is related to the disorder itself. One hypothesis is that the FA-A gene may be involved in a developmentally important function which could involve a number of diverse cellular systems. The FA-A gene product could play a role not only in DNA repair but also in other cellular processes critical to development and differentiation, such as transcription and DNA replication. A defect in such a protein could thus affect hematopoiesis, leukemogenesis, and organ and cytoskeletal development via its pleiotropic effects on these different cellular processes.

References

1. Lambert, M.W., Tsongalis, G.J., Lambert, W.C., Hang, B., and Parrish, D.D. 1992. Defective DNA endonuclease activities in Fanconi's anemia cells, complementation groups A and B. Mutation Res. 273: 57-71.

2. Lambert, M.W., Fenkart, D., and Clarke, M. 1988. Two DNA endonuclease activities from normal human and xeroderma pigmentosum chromatin active on psoralen plus ultraviolet light treated DNA. Mutation Res., 193: 65-73.

3. Hang, B., Yeung, A.T., and Lambert, M.W. 1993. A damage-recognition protein which binds a DNA containing interstrand crosslinks is absent or defective in Fanconi anemia, complementation group A cells. Nucleic Acids Res., 21: 4187-4192.

4. Kumaresan, K., Hang, B., and Lambert, M.W. 1995 Human endonucleolytic incision of DNA 3' and 5' to a site-directed psoralen monoadduct and interstrand cross-link. J. Biol. Chem. 270, in press ••

Buchwald - cantinued from page 12

that the FA-C gene is expressed in tissues that are affected in human patients. For example, the FA-C gene is expressed in the developing bone, kidney, lung and blood. In particular, we see the gene being expressed in cells that are both rapidly growing and are precursors of other cells. In this respect, the pattern of FA-C expression is consistent with what we think the FA-C protein does: it protects cells from damage during critical periods.

Results on a mouse model developed by gene targeting. In this procedure, molecular genetic methodologies are used to disrupt the mouse equivalent of FA-C (usually referred to as Fac). We disrupted the middle of the gene, predicting that this would produce a non-functional protein. To confirm this supposition, we tested the cells from the mutant mice for their sensitivity to diepoxybutane (DEB) and mitomycin C (MMC), the two compounds used to confirm the diagnosis of FA. We found that, indeed, the cells from the mutant mice were extremely sensitive to these two drugs, in effect mimicking the human condition. Unlike patients, however, the mutant mice had no congenital malformations and did not develop bone marrow failure during the first six months of age. (Mice are mature at six months; they reach reproductive age by six weeks.) We are following the mice as they age, to determine if they will develop bone marrow failure at a later date.

The most dramatic result observed is that both male and female mutant mice have problems with fertility, which become more serious as the mice age. In the mouse strains that we have been working with, about 40% of male mice are infertile at 3 months of age and 67% are infertile by about 9 months of age. It appears that the problem with these mice is degeneration of their capacity to produce sperm. With regard to the females, about 67% are infertile at 3 months and 80% at 9 months. In this case the problem appears to be similar, in that the mice have problems producing eggs. Thus, in both males and females, alteration of Fac leads to problems in germ cell (sperm and egg) production. Infertility in FA patients has also been noted. Thus, the mutant mice share some of the characteristics of the human patients and not others.

Our future experiments will be devoted to studying mutant mice as they age, to stress the mice with various treatments that may exacerbate the bone marrow failure and to breed different strains of mutant mice, to see if we can uncover other genetic influences on the severity of the clinical problems. If we can develop a suitable clinical picture, we will then attempt to test gene therapy in the mice. ••

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D9S196, FA-Ci11, D9S753, D9S180 and D9S176. Our analysis showed strong evidence for a founder effect for the IVS4 mutation. Linkage disequilibrium was found between the IVS4 mutation and a polymorphism in intron 11 of the FA-C gene; 100% of affected chromosomes had the same allele for this polymorphism. Haplotype analysis with the other markers provided additional evidence for a founder effect for the IVS4 mutation within the Ashkenazi Jewish population. We are currently analyzing these data in order to estimate the age of this mutation in the Jewish population.

In a separate study, we have linked a major locus causing FA to marker D16S520 (16q24.3) by homozygosity mapping in 23 inbred families from the International Fanconi Anemia Registry (IFAR). This method is based on the assumption that if a child of consanguineous (closely related) parents is affected with a rare genetic disorder, that child received an identical copy of the disease allele from each parent. In addition, DNA markers tightly linked to the disease locus would also be homozygous (have identical alleles at that locus) by descent. Although the probability that any given marker will be homozygous by descent in an inbred individual is high, the chance that two unrelated inbred individuals will be homozygous by descent at the same marker is considerably lower. By locating regions of marker homozygosity among different inbred individuals, one can determine where a disease-causing gene resides. Somatic cell hybrid analysis to determine complementation group was not performed on our patients, but families known to belong to FA complementation group C by previous linkage studies or by mutation analysis were excluded from our family set. We did not see evidence of linkage at D16S422 which is 14 cM proximal to D16S520; therefore the FA gene is distal to D16S520. This result independently replicates the linkage result reported here by Arwert and Mathew; together they provide strong evidence for an FA locus near the telomere of the long arm of chromosome 16. Approximately two-thirds of our families showed linkage to D16S520. Significant evidence of genetic heterogeneity was found in our study. Simultaneous search analysis identified several additional potential loci that could account for a small fraction of Fanconi anemia families. Further linkage studies are being carried out to identify the location of these other genes in our IFAR linkage family set. ••

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Although not detailed here, the results in these first 7 patients suggest that the Minnesota regimen of Cytoxan (40 mg/kg total dose) and TBI 450 cGy is tolerable and effective in eliminating leukemia. However, graft failure and severe GVHD are substantial barriers to the success of unrelated BMT.

T Cell Depletion of Unrelated Bone Marrow by Counterflow Elutriation.

Counterflow elutriation is one of several potential methods of removing the T cells (which cause GVHD) from the bone marrow. This method has been used in Europe and the US in more than 400 patients with sibling donors. As a result of the high risk of GVHD in patients with unrelated donors, as reported by Dr. Davies above, we initiated studies to determine the effectiveness of elutriation in preventing GHVD after unrelated BMT in patients with specific genetic diseases (n=21) or leukemia (n=23). Thus far, we have demonstrated engraftment in all but two with only one patient developing grade III-IV GVHD.

New Treatment Protocol for Patients with FA.

On the basis of the international data and results at the University of Minnesota presented above, we propose to use the combination of Cytoxan 10 mg/kg/day for 4 days, total body irradiation (450 cGy once) and antithymocyte globulin (15 mg/kg twice a day for 5 days) as a tolerable and potentially efficacious preparative therapy in patients with Fanconi anemia. In an attempt to reduce the risk of severe GVHD, marrow will be T cell depleted by counterflow elutriation; and, in an attempt to recover as many stem cells (the parent cells responsible for marrow engraftment) as possible, the lymphocyte-containing fractions will be passed over a CellPro CEPRATE™ SC system. Thus far, 6 patients with matched and mismatched unrelated (n=4) or related (other than HLA identical siblings) donors (n=2) have been treated in this manner. Notably, all patients tolerated the preparative therapy and all engrafted. Unfortunately, 2 patients with unrelated donors lost their graft after transient recovery. Of the 4 long-term engrafted patients, none have had GVHD. For future patients, it is likely that the treatment protocol will be modified in an attempt to reduce further the risk of graft failure.

Protocol Eligibility.

(1) Patients must be under 55 years of age with a diagnosis of FA (confirmed by diepoxybutane [DEB] chromosomal breakage testing).

(2) Patients must have severe pancytopenia and be transfusion dependent despite androgen or growth factor therapy or myelodysplastic syndrome with persistent (two marrow tests four months apart) clonal chromosomal abnormalities or leukemic transformation.

(3) Patients must have either a serologically HLA-A, B, DR matched or one antigen mismatched related (but not a sibling) or unrelated donor.

(4) Patients must have good lung, heart, kidney and liver function.

(5) Patients must be available for follow-up evaluations at 30, 60, 100, and 180 days post BMT and yearly for 3 years.

Donor Search Procedure.

Patients referred to the University of Minnesota for unrelated BMT, regardless of the disease, will have searches of both the marrow and cord blood registries. If the cord blood is considered to be suitable on the basis of HLA typing and number of cells, it may be used in preference to marrow since it may be immediately available. Patients and family members will be advised of their choices of donor type. Use of cord blood does not alter the treatment plan.

Cord Blood Transplant Results.

An alternative source of stem cells includes umbilical cord blood. This blood which is normally discarded after a baby is born, can be frozen and stored for use in transplantation. It has already been shown that umbilical cord blood contains sufficient numbers of stem cells to engraft patients weighing less than 50 kg, and early information suggests that it is sufficient for larger recipients as well. Thus far, 4 patients with Fanconi anemia have been transplanted with umbilical cord blood from mismatched unrelated donors. Notably, 3 of the 4 are alive 3-14 months after transplantation with little GVHD. Longer follow up is required before routinely recommending this source of stem cells as a first choice; however, it should be considered as an option for all patients. Except for the source of stem cells, the patients' treatment would be virtually identical to that used in patients with bone marrow donors. →

Summary.

Bone marrow transplantation is the *only proven definitive treatment* for the hematologic abnormalities of FA. All patients should be seen regularly by a *hematologist,* with bone marrow evaluations at least every 2 years when the blood counts are satisfactory. This is the only way to determine the natural history of the disease, in terms of time to development of myelodysplastic syndrome and leukemia. This knowledge will allow us to optimize the various treatment approaches currently available: gene therapy, growth factor therapy, bone marrow transplantation, etc. Once the blood counts decline and especially after the diagnosis of myelodysplastic syndrome, bone marrow evaluations must be performed every 4 months. A major goal of all interested parties should be the development of a treatment schema for all patients that will direct patients and families to appropriate therapies for the specific nature of the hematologic abnormality at a given time. Patients with HLA-identical sibling donors should be considered for BMT early after the onset of signs of impending bone marrow failure. Patients without sibling donors should attempt to put off an alternate donor BMT for as long as possible while we and others try to optimize this treatment approach. However, waiting until the onset of leukemia or infection, or after multiple transfusions, is not optimal; this will only guarantee a lower chance of success with BMT. Once there are signs of bone marrow failure or myelodysplastic syndrome, a BMT center should be identified. Searches of the marrow and cord blood registries should be initiated. Beginning a search to determine what's available does not commit you to immediate BMT therapy. Searches take an average of 4–5 months, time that may be crucial for survival. Patients referred late, after the onset of refractory severe neutropenia (with high risk of infection), after the onset of refractory anemia and thrombocytopenia (with a large transfusion requirement) and after the onset of leukemia, may not survive long enough to complete the search.

One potential treatment approach has been outlined above. Whether this approach is optimal or even satisfactory remains to be determined. However, to determine more quickly the best treatment for patients with FA, the following transplant centers worldwide are now working together on this project:

Hôpital St Louis: *Eliane Gluckman, MD*
University of Minnesota: *John Wagner, MD, Stella Davies, MD*
Children's Hospital of Cincinnati: *Richard Harris, MD*
University of Leiden: *Jaak Voss, MD*
Medical College of Wisconsin: *James Casper, MD*
Duke University Medical Center: *Joanne Kurtzberg, MD*
Memorial Sloan-Kettering Cancer Center: *Al Gillio, MD*
Fred Hutchinson Cancer Research Center: *Joachim Deeg, MD*
Children's Hospital - Boston: *Eva Guinan, MD*
Children's Hospital of Los Angeles: *Gaye Crooks, MD* ••

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The first set of studies revealed that the vector DNA sequences were detected predominantly in the liver tissue, suggesting that AAV may possess organ-tropism for liver. The second set of studies documented high-frequency transduction and short-term expression of the transduced reporter gene in spleen colonies. The third set of studies indicated transduction and long-term expression of the human globin gene in bone marrow, but not in thymus, of these mice. Since no adverse effects were evident following either direct viral injection, or reconstitution with the recombinant AAV-infected cells, these studies suggest that AAV vectors may prove potentially useful for human gene therapy. The identification and characterization of the putative cellular receptor for AAV also has important implications in the potential use these vectors in human gene therapy. The development of the AAV–B19 hybrid vector system promises to lead to potential gene therapy of human hemoglobinopathies in general, and sickle-cell anemia and β-thalassemia in particular. The AAV vectors also promise to provide a potentially safe and effective alternative for successful gene therapy for Fanconi anemia. ••

Joenje - continued from page 6

must be killed by the selective agents. Two types of genetic marking may be used: (1) both fusion partners are marked with different drug-resistance markers, or (2) one fusion partner is doubly marked with both a dominant resistance marker and a recessive sensitivity marker. A doubly marked cell line has the distinct advantage that hybrids prepared from this cell line can be selected after fusions with any other UNMARKED cell line. This means that the second partner does not need to be marked prior to the fusion experiment. This is a critical advantage since genetic marking of FA cells has proved to be exceedingly difficult.

In collaboration with Buchwald we were able to generate doubly marked derivatives from cell lines representing the groups FA-A – FA-D. These "reference cell lines" are now being used as a panel of fusion partners in a more extensive complementation study aiming at the analysis of 100 patients. The main purpose of this study is to assess the number of major complementation groups existing in FA. The patient population is recruited from many different regions in Europe, in order to maximize the possibility of finding new complementation groups.

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Results

The analysis of 40 European FA patients has now been completed. Together with the 7 patients characterized before in Buchwald's laboratory, the results are: FA-A: 31 patients (66%), FA-B: 2 (4.3%), FA-C: 6 (12.7%), FA-D: 2 (4.3%). *Cell lines from at least 6 patients were found to complement all 4 reference cell lines and were therefore classified as belonging to a fifth class, FA-E.* This class was defined only as "different from A, B, C and D" and *could thus include even more groups.*

Are there more than five FA genes?

To determine whether the E group contains any additional groups, individual E cell lines must be genetically marked, to allow fusions among the various members of this group to be analyzed. We were recently able to mark a number of E cell lines so that further analysis of the E group patients has become feasible. The first results indicate that fusions among several of the E group cell lines all complement. Based on previous reasoning, this would imply that these cell lines belong to different complementation groups, *bringing the total number of groups to 7 or 8.*

A word of caution:

This result may reflect the real situation, but there is a caveat. We have seen two examples of cell lines from FA-A patients that yielded contradictory results: among multiple parallel fusions with the reference group A line there were MMC-hypersensitive (non-complementing) hybrids, confirming the A type, as well as hybrids that were clearly MMC-resistant, indicating a non-A type. Based on genetic linkage and other pedigree data these families had to be type A, so the non-A results were somehow in error. We believe that the apparently "false" non-A result is based on one of the mutations in the FA-A gene "reverting" to normal. Examples of genetic reversion in cell lines has been documented in other diseases similar to FA, such as ataxia telangiectasia, Bloom syndrome, and xeroderma pigmentosum. For xeroderma pigmentosum cell lines it has been documented that genetic reversion is actually *stimulated* by cell fusion.

Thus, there is a possibility that the patients so far classified as FA-E in fact represent FA-A, B, C, or D patients whose cell lines complemented due to genetic reversion rather than true complementation. Additional work needs to be done to establish the status of each individual patient who has now been classified as FA-E.

Given the pitfall of genetic reversion, which needs to be carefully addressed, what can be said about the minimum number of distinct FA-causing genes? *Since the genes for groups A, C, and D have now been mapped to 3 distinct chromosomal locations* (see corresponding abstracts), *these complementation groups must represent distinct FA genes.* The status of the B and E group patients remains uncertain. Ultimate proof for the existence of a distinct FA gene comes from its identification at the DNA level and the finding of inactivating mutations in FA patients. Until then we can say only that *"the minimum number of FA genes is 3, while there is evidence for the existence of 7 or 8".* ••