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Special Update

Gene Therapy for Fanconi Anemia

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As part of a larger initiative aimed at advancing research in the field of gene therapy (the Harvard Gene Therapy Initiative) and in collaboration with several investigators including Eva Guinan M.D., Richard Mulligan Ph.D., and Colin Sieff M.D., we have initiated a program aimed at establishing clinical gene transfer trials for patients with Fanconi anemia type A. Gene therapy is performed by placing the Fanconi gene into a vector, or piece of DNA designed to carry and express a gene. This vector is placed or "packaged" into a viral particle that infects a cell and deposits the DNA. After being deposited in the cell, the vector DNA containing the FA gene then joins the chromosomes of the cell, becoming an integral part of the DNA of the cell. When the cell divides, the vector DNA divides with the chromosomes of that cell, and all the cells derived from the first corrected cell will have copies of the vector FA gene. We are using a modified Moloney-based retroviral vector engineered for increased stability and long-term expression (pMMP). With this pMMP-FAA vector, we have obtained successful gene correction of a number of cell lines and patient bone marrow samples. When compared to infection with a Fanconi type C retrovirus, bone marrow cells from Fanconi type A patients have shown markedly increased growth after gene correction. This result is similar to what has been shown in FA type C, and is encouraging because it suggests that gene corrected bone marrow progenitor cells may have a survival advantage over uncorrected marrow. Repopulation of the marrow by a relatively small number of gene-corrected bone marrow cells may be possible.

Gene therapy into human bone marrow "stem cells" has been largely unsuccessful. The main obstacle has been that, while investigators have been able to successfully transfer genes into a high percentage of short-term bone marrow progenitor cells, it has been very difficult to transfer genes into the long-term repopulating marrow cells or stem cells. In clinical gene therapy trials to date, the presence of cells containing the transferred gene in treated patients has fallen to well less than 1% within a year of treatment. In upcoming trials, we hope to improve the rate of gene transfer into the long-term repopulating bone marrow stem cells by several methods. First, we are exploring the use of vesicular stomatitis virus type G (VSV-G) viral capsids for our gene transfer vehicle. This type of virus has two advantages. VSV-G has a broad range of infectibility, being able to effectively transfer genes to many different types of cells. This may mean that it will be able to get into human bone marrow stem cells better than other retroviruses used previously. In addition, this type of virus has a unique characteristic in that we can superconcentrate it. By using superconcentrated virus, we may have a better chance of transferring the gene into human stem cells. Whether or not this approach will be better awaits upcoming human trials, but we have some promising preclinical data. By looking at long-term bone marrow cultures and a human cell into mouse model (two of the best ways we have to measure very early human bone marrow cells), we see gene transfer in a high percentage of the early human marrow cells.

Another aspect of improving the efficiency of gene transfer involves optimizing the conditions under which the gene transfer occurs. Currently, in order to infect the cells with the vector-containing viruses, bone marrow cells are grown outside of the body in cell "cultures" for several days. We have good evidence from our mouse model to suggest that extended culture of marrow cells outside the body may decrease the number of marrow stem cells. We are therefore currently working to shorten the amount of time needed for gene transfer to an optimal point. Also, Fanconi anemia bone marrow is especially sensitive to damage by a number of factors associated with cell culture and the gene transfer process such as exposure to high levels of oxygen. We hope to create cell culture conditions that will protect the character of the marrow and preserve the stem cells while maximizing gene transfer into these cells. In order to find the best conditions for gene transfer, we have been obtaining bone marrow samples from FA patients on a voluntary basis. We now have a protocol open that allows voluntary donation of enough bone marrow from a given patient to be able to study a number of cell culture and gene transfer conditions. We are especially interested in FA patients who are early in their disease process, or who have relatively mild bone marrow disease, since their marrow may have larger numbers of stem cells.

We are currently in the process of completing a formal outline of our FA type A treatment plan. Once completed, this gene therapy protocol will need first local and then national review. We have recently completed a treatment grade vector facility and we are currently starting to produce a grade of retrovirus that can be used for human clinical trials. Depending upon the extent of the regulatory process, we should be able to offer clinical gene therapy for patients with FA type A during the next 6–24 months.

FAA Mutations in Italian Patients Including Two Large Genomic Deletions of the Gene

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In order to characterize mutations affecting the FAA gene, we have screened all exons and the promoter region of 57 Italian Fanconi anemia patients by RNA-SSCP. Eleven mutations, all but one expected to cause a premature truncation of the protein, have been identified. Three different C>T transitions at position 790, 2005, 2314 determined the substitution of a glutamine with a stop codon in exons 8, 22 and 24, respectively. Four putative splicing mutations were identified in the donor splicing site of introns 9, 10, 14 and 28. In order to verify the alternations at the RNA level, patient cDNAs were also analyzed. Moreover, two patients had an insertion of a single G residue in exon 36, another two carried a duplication of 19 bp in exon 29 and patient FA41 had a CT deletion in exon 37. We have also found different amino acid substitutions and variants that were common polymorphisms as determined by amino analyzing chromosomes from healthy individuals. However, the D1359Y variant is likely to be a mutation. It was not detected in non-FA chromosomes and on the basis of a calculated prediction, it altered the local hydrophobicity of the FAA protein.

Consistent with linkage and haplotype analysis in Italian pedigrees, which revealed a high frequency of compound heterozygotes and a wide variety of haplotypes, different mutations characterize the Italian FA alleles, and none of these seems to occur at a high frequency.

Surprisingly, the screening of all 43 exons of the FAA gene has allowed us to identify only a few mutations. We think that genomic deletions could affect the FA alleles in several patients. Evidence for deletions has previously been reported in both papers describing the cloning of the FAA gene. In addition, two Italian patients, apparently homozygous for a mutation, inherited the mutation from only one parent, suggesting that the other FA allele is likely to be a genomic deletion. Moreover, in patient VU337, a deletion of four exons was detected in the cDNA. Amplification of these exons failed to produce a PCR product, suggesting that this patient was either homozygous for a genomic deletion of this region, or had another mutated allele from which these exons are also deleted. The last hypothesis seems to be correct because FISH analysis of metaphase chromosomes showed that in one FA allele the FAA gene is completely deleted. The other allele resulted in a genomic deletion, maternally inherited, of approximately 5.0 kb including exons 18 to 21, as determined by Southern blot and sequence analysis of a PCR product from genomic DNA using specific primers on exons 17 and 22.

It might be that some factors such as an intrinsic instability in the FAA region might lead to genomic deletions or other structural rearrangements of the gene. Since screening based on the analysis of single exons by genomic DNA amplification apparently detects only a minority of the mutations, other methods designed to detect alterations in the genomic structure of the gene or in the FAA polypeptide may be helpful in the identification of FAA mutations.

Complementation Studies in Fanconi Anemia: an Update

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The most important cellular characteristic that distinguishes FA cells from non-FA cells is a high sensitivity to killing by a class of chemical compounds known as "crosslinkers." Examples of such compounds are mitomycin C (MMC), diepoxybutane, cyclophosphamide, and cisplatin. When lymphoblastoid cell lines derived from unrelated patients are fused, hybrid cell lines are obtained that can be grown indefinitely and tested for crosslinker sensitivity. It appears that such hybrids show either MMC resistance (in which case the defect is "corrected" or "complemented") or MMC sensitivity (lack of complementation). Patients whose cell lines do not complement each other in a fusion hybrid are said to belong to one and the same "complementation group." Patients within one group are thought to have mutations in the same gene. By analyzing hybrid cell lines derived from a large number of patients, one can obtain an estimate of the number of complementation groups and hence the number of existing FA genes. Thus far as many as 8

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Mutation Analysis of Fanconi Anemia FAC and FAA Genes

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Arleen Auerbach

The International Fanconi Anemia Registry (IFAR) was established at The Rockefeller University in 1982 to collect clinical and genetic information from a large number of Fanconi anemia (FA) patients. For a study of the clinical effects of specific mutations in the FA complementation group C gene (FAC), we used ARMS assays to screen genomic DNA from 397 FA patients in the IFAR for six FAC mutations we had found in a prior screen of IFAR patients using SSCP. At least one mutated FAC allele was identified in 59 (14.9%) FA patients. The most common FAC mutation is IVS4 +4 A>T, found in patients of Ashkenazi Jewish ancestry. Less common mutations included in this screen were 322delG, Q13X, R185X, R548X and L554P. FA-C patients were divided into three subgroups based on results of a genotype-phenotype analysis using the Cox proportional hazards model: (1) patients with the IVS4 mutation (n=26); (2) patients with at least one exon 14 mutation (R548X or L554P) (n=16); and (3) patients with at least one exon 1 mutation (322delG or Q13X) and no known exon 14 mutation (n=17). Kaplan-Meier analysis shows that IVS4 or exon 14 mutations define poor risk subgroups, as they are associated with significantly earlier onset of hematologic abnormalities and poorer survival compared to exon 1 patients and to the non-FA-C IFAR population. Sixteen of the 59 FA-C patients (27%) have developed acute myelogenous leukemia (AML). There was no direct correlation between the degree of cellular hypersensitivity to the clastogenic effect of DEB and severity of clinical phenotype (Gillio et al., Blood 90:105, 1997).

We have recently completed a screen of genomic DNA from a total of 97 racially and ethnically diverse FA patients from the IFAR for mutations in the FAA gene. The complete coding sequence (43 exons) was amplified from genomic DNA and screened by single-strand conformational polymorphism (SSCP) analysis. A total of 77 variant bands were detected and sequenced. Forty-one of the variants are probably benign polymorphisms. Amplification refractory mutation system (ARMS) assays developed for eight of these common polymorphisms simplify their detection and can be used to map other genes in this region of chromosome 16q. Thirty-six of the variants are likely to be pathogenic mutations, including frameshift, splicing, nonsense, and missense mutations. Mutations fell into two classes, either base substitutions or microdeletions/microinsertions. The latter were usually associated with short direct repeats of 2-5 nucleotides, either in tandem or separated by intervening nucleotides. This type of mutation is thought to be generated by a mechanism of slipped-strand mispairing (SSM) during DNA synthesis. Two of the microdeletions, 1115-1118del and 3788-3790del, account for 2 and 5% of the FA alleles in a panel of 350 non FA-C probands from the IFAR. 3788-3790del appears in a variety of ethnic groups, and is found on at least two different haplotypes. We conclude that FAA is highly polymorphic, and may be hypermutable. SSM, a mutational mechanism recognized as important for the generation of germ-line and somatic mutations in a variety of cancer-related genes, may be a major mechanism for FAA mutagenesis.

A Fanconi Anemia Mutation Database has been set up on the World Wide Web. Co-Curators of the database are Arleen D. Auerbach and Christopher G. Mathew. Peter C. Verlander is the database manager. The URL for the Fanconi Anemia Mutation Database is: <http://www.rockefeller.edu/fanconi/mutate>.

Mutation Screening of the FAA Gene

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Mutations in the Fanconi anemia complementation group A gene cause about 60–65% of FA cases worldwide. The gene comprises 43 exons over 80 kb of genomic DNA, with the exon sizes varying from 34 to 188 bp. The major transcript from the FAA gene is of about 5.4 kb, but a complex picture of alternative splicing of this gene is beginning to emerge; these alternatively spliced forms may or may not have biological significance. Some of these alternate transcripts may contain exons not described in the initial cloning papers and not included in database entries for FAA.

We have been applying a wide range of screening techniques to the search for mutations in the FAA gene, including Southern blotting, SSCP, RT-PCR, direct sequencing, fluorescent chemical cleavage of mismatch (FCCM) and fluorescent dosage

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Overview of the Genetics of Fanconi Anemia and the Cloning of the Genes

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The analysis of Fanconi anemia (FA) has been guided by its genetic basis since the first description by G. Fanconi, who recognized the disease was inherited as an autosomal recessive trait. A more complete and definitive analysis by Schroeder et al. in the mid 1970s confirmed that the disorder was, indeed, autosomal recessive.

Genetic analysis of somatic cell fusion products (heterokaryons) led to the first description of genetic heterogeneity in the early 1980s. This was followed by a more reproducible analysis based on lymphoblast hybrids. The current classification of the genetic basis of FA, including the nomenclature used, is based on these studies (described in more detail in the talk by Dr. H. Joenje). The complementation studies led to the realization that FA could be caused by defects in several genes.

A variety of biochemical and cellular approaches have been used to attempt to identify the basic defect in the disease, with the longer term aim of finding the defective genes. These studies attempted to identify a defective function in one of the various pathways thought to underlie the principal clinical or cellular features of the disease (DNA repair, mutagenesis, growth control). However, none of these was successful and it required a genetic approach to lead to the identification of the FA genes.

Genetic approaches were based on attempts to find the FA genes by complementation of the principal cellular feature: increased sensitivity to cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB). Initial attempts aimed to reproduce methods successfully used to complement rodent cell lines. Genomic DNA was introduced into MMC- or DEB-sensitive cells that were then subjected to selection in one or the other drug. Resistant cells presumably had integrated the "normal" version of the gene; subsequent cloning of the genomic DNA of the transfectants would lead to the identification of this gene. Unfortunately, human cells integrate much less genomic DNA than rodent cells and it was not possible to use this method to find FA genes.

FA genes were, however, successfully cloned by the use of episomal cDNA libraries, since this method obviated the difficulties in introducing DNA into human cells. In this manner, first FAC (the gene defective in complementation group C), and subsequently FAA were isolated. FAA was also isolated by positional cloning using a group of FA patients classified into complementation group A, the most prevalent group.

As will be described in other talks, FAA and FAC code for unusual proteins, not related to known entities, thus complicating our understanding of their cellular role and the basis of the disease phenotype. Current research in various laboratories is aimed at identifying the other FA genes, using various approaches. More specifically, FAD has been located to 3p22-26 by microcell mediated gene transfer; efforts are underway to clone this region and identify the gene. Nothing is known about the chromosomal location of any of the other FA genes.

FAA and FAC Gene Structure and Transcription

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The genomic structure of the FAA and FAC genes has been resolved by using genomic clones obtained from different human libraries. The FAA gene contains 43 exons spanning approximately 80 kb. Exons range from 34 to 188 bp and introns from 88 to 7000 bp. The FAC gene contains 14 exons and two alternative sequences in the untranslated region of the gene. We have estimated that FAC gene is at least 150 kb in size. Knowledge of the structure of the FAA and FAC genes provides an invaluable resource for the discovery of mutations in these genes that account for about 75% of the Fanconi anemia patients. In the FAA gene, alternative sequences have also been identified in clones representing different forms of the FAA messenger (mRNA). Three new exons called 10a, 28a, and 29a can in fact be detected in several tissues. The FAA mRNA heterogeneity is generated by a mechanism of alternative splicing and assembly of different exon units. The presence of several variants raises the question of whether the alternatively spliced transcripts have significant biological functions.

FAA and FAC genes appear to be ubiquitously expressed in both embryonic and adult human tissues even though significant differences at the expression level have been detected. The ubiquitous expression of the FA genes is expected for genes coding for proteins assumed to have a general cellular function. However, developmental abnormalities seen in patients have not yet been correlated. Thus, as part of a long-term goal of understanding the function of FA proteins and the role of mutated genes in Fanconi anemia, we have been studying the mechanisms that control the expression of FAA and FAC genes. The transcriptional regulation of the FAC gene is complex not only for the presence of two putative regulatory regions but also for the identification of sequences with positive and negative control on the expression. Similarly, a functional analysis of the FAA promoter is in progress and will provide advances in the knowledge of the expression of this gene.

GENE PRODUCTS: DNA DAMAGE

A Defective Damage-Recognition Protein With Specificity for DNA Interstrand Cross-Links in Fanconi Anemia Complementation Group A Cells

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Cells from patients with Fanconi anemia (FA) have increased sensitivity to DNA interstrand cross-linking agents and a reduced ability to repair DNA damage produced by these agents. We have previously shown in FA complementation groups A (FA-A) and D (FA-D) cells that this defect is related to decreased ability of a protein complex, with specificity for DNA interstrand cross-links, to incise damaged DNA (Mutat. Res. 273:57, 1992). Introduction of this complex into FA-A and FA-D cells treated with 8-methoxypsoralen plus UVA light, an agent that produces DNA interstrand cross-links, resulted in correction of their repair defect (Biochem. Biophys. Res. Commun. 230:587, 1997). In FA-A cells, this repair deficiency may be related to a defect in a protein that specifically recognizes damage in DNA (Nucleic Acids Res. 21:187, 1993). Studies have now been carried out to identify the proteins that recognize and bind to sites of interstrand cross-links in DNA. A short fragment of DNA was synthesized and interstrand cross-links were formed in it using another agent, 4,5',8-trimethylpsoralen (TMP) plus UVA light. Protein extracts from the nuclei of normal human cells, which contained the complex with specificity for DNA interstrand cross-links, were incubated with the TMP cross-linked DNA. Using this method, three proteins were identified that specifically bound to the damaged DNA. One of these was a relatively large protein (approximately 230 kDa). In separate experiments, we have developed monoclonal antibodies (Mabs) against a 230 kDa protein present in the normal protein complex that has specificity for interstrand cross-links. Evidence indicates that this protein is involved in the repair of DNA interstrand cross-links and that this is the same protein that binds to TMP cross-linked DNA. These results further strengthen the view that there is a defect in a protein that specifically recognizes damage (i.e., DNA interstrand cross-links) in FA-A cells. Studies are in progress to determine whether transfection of FA-A cells with the FA-A cDNA (obtained from Dr. Emanuel Buchwald) corrects the deficiency in this 230 kDa protein in these cells.

Functional Studies of the FA-C Protein

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Little is known about the function of FAC, the cytoplasmic protein encoded by the Fanconi anemia (FA) group C gene. As FA cells are susceptible to mitomycin C, we considered the possibility that FAC might interact with enzymes involved in the bioreductive activation of this drug. Here we report that FAC binds to NADPH cytochrome-P450 reductase, a microsomal membrane protein involved in electron transfer. FAC-reductase interaction requires the amino-terminal region of FAC and the cytosolic, membrane-proximal domain of the reductase. This domain contains known binding sites for flavin mononucleotide and cytochrome c. Addition of either cofactor to cytosolic lysates inhibits FAC-reductase immune complex formation in vitro. Aside from a physical interaction, FAC also appears to be functionally coupled to this enzyme as its expression in COS-1 cells suppresses its ability to reduce cytochrome c in the presence of NADPH. We propose that FAC attenuates the activity of NADPH cytochrome-P450 reductase in vivo by competition with one or more cofactors.

We have also identified a genetic pathway that is altered in FA. Using cDNA differential display, we isolated MxA, an interferon-inducible gene that is highly upregulated in cells of FA complementation groups A, B, C and D. MxA expression is suppressed in FA group C cells complemented with wild-type FAC cDNA as well as in non-FA cells. A posttranscriptional mechanism rather than transcriptional induction appears to account for MxA overexpression. Forced expression of MxA in Hep3B cells enhances their sensitivity to mitomycin C and induces apoptosis, similar to the FA phenotype. Thus, MxA is a downstream target of FAC and is the first genetic marker to be identified among multiple FA complementation groups. These data suggest that FA subtypes converge onto a final pathway, which is intimately related to the interferon signaling mechanism. Constitutive activity of this pathway may explain a number of the phenotypic features of FA, particularly the pathogenesis of bone marrow failure.

Subcellular Localization of the Fanconi Anemia Group A Protein is Cell Type-Dependent

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Frank Kruyt

The protein encoded by the recently cloned FA complementation group A gene, FAA, has been expected to localize in the nucleus as based on the presence of sequences homologous to a bipartite nuclear localization signal (NLS) and a leucine repeat motif. In contrast to this expectation, we have shown that a functionally active FAA-green fluorescent protein (GFP) hybrid resides in the cytoplasmic compartment of human kidney 293 cells. In accordance with this finding, disruption of either of the two putative motifs by site-directed mutagenesis failed to affect both subcellular localization and the capacity to complement hypersensitivity to the cross-linking agent mitomycin C in FA-A lymphoblasts. In addition, fusion of the SV40 Large T NLS to the amino-terminus of FAA-GFP did not result in enforced nuclear targeting of the chimera, indicating that FAA is somehow maintained in the cytoplasm of 293 cells via presently unknown mechanisms. Thus, like the first identified FA protein, FAC, FAA seems to exert its function in the cytoplasmic compartment in these cells.

To further explore the subcellular localization of FAA, we generated a rabbit polyclonal antibody against the bacterially expressed amino-terminal portion of FAA, encompassing amino acids 2-321, fused to GST. Characterization of the affinity-purified polyclonal antibody in immunoblotting and immunoprecipitation experiments using total cell extracts derived from FAA-expression vector-transfected COS-1 cells showed one major specific band at a molecular weight of approximately 165 kDa, matching well the predicted size of FAA, indicating that the antibody specifically detects FAA and that the protein apparently lacks extensive post-translational modification. Interestingly, however, in immunofluorescence microscopy assays on FAA-expression vector-transfected COS-1 cells, the antibody demonstrated primarily nuclear staining. This unexpected finding may indicate that the subcellular localization of FAA is dependent on the cell type investigated. Experiments are in progress to further examine the subcellular localization of FAA in various cell types, using either the generated antibody or GFP-tagged FAA chimera, and upon treatment with various apoptosis-inducing agents.

The Fanconi Anemia Group A Protein Localizes Primarily to the Nucleus

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Fanconi anemia (FA) is an autosomal recessive disorder characterized by skeletal abnormalities, bone marrow failure, and predisposition to leukemia. Cells from FA patients display marked chromosomal instability and are hypersensitive to DNA-damaging agents such as mitomycin C and diepoxybutane. The heterogeneous nature of FA is such that five genetic complementation groups, termed A–E, have been identified. A cDNA for group A (FAA) was recently cloned and shown to encode a protein with molecular weight of approximately 162 kd. As a preliminary step to investigate the function of the FAA protein, the cellular localization of FAA was determined. FAA fused to the green fluorescent protein (GFP) was transiently expressed in COS-7 cells, which were visualized by fluorescence microscopy after 24 hours and 48 hours. At both time points, fluorescence was most intense in the nucleus, and fluorescence in the cytoplasm was substantially weaker. Confocal imaging of COS-7 cells expressing the GFP-FAA fusion protein confirmed that fluorescence was distributed throughout the nucleus, but was absent from the nucleoli.

Expression of the GFP-FAA fusion in an FA-A cell line, HSC99, also revealed a primarily nuclear localization of FAA. In addition, when FAA tagged with the FLAG peptide epitope was expressed in COS-7 cells and detected by immunostaining using an anti-FLAG antibody, the FLAG-FAA protein was predominantly nuclear and weakly cytoplasmic. FAA has two overlapping bipartite nuclear localization signals (NLS) at the N-terminus between residues 18–34 and 19–35. To determine if these NLS motifs were functional, a GFP fusion of a mutant FAA protein lacking the N-terminal 39 amino acids (FA39) was transiently expressed in COS-7 cells. Twenty-four hours following transfection, GFP-FA39 was found only in the cytoplasm. However, 48 hours following transfection, approximately half the cells showed equal fluorescence in both the nucleus and the cytoplasm, suggesting that removal of these motifs delays the import of FAA into the nucleus. Localization of FAA to the nucleus may indicate a direct role of FAA in the maintenance of DNA stability. (Supported by MRC and NIH.)

Overview on DNA Repair

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Defective DNA repair has been hypothesized as a possible mechanism for bone marrow failure and progression to leukemia in Fanconi anemia patients. Although it is not yet clear whether any of the FA gene products is indeed involved in DNA repair, it is important to consider the emerging information of these gene products in terms of their possible involvement in such pathways. In order to set the scene for this, an overview will be presented of the various strategies that cells take to eliminate damage from their genome. Since FA cells are characteristically sensitive to DNA crosslinking agents, particular attention will be paid to the DNA repair mechanisms that have been postulated to be involved in the repair of damage caused by these agents.

Cells employ three distinct strategies for DNA repair: (i) the direct reversal of DNA damage; (ii) the excision of damaged DNA segments followed by resynthesis of the excised region; and (iii) the rearrangement of DNA strands by recombination to alleviate the toxic effects of certain lesions. These strategies will be outlined, again paying particular attention to those relevant to DNA crosslink repair. It should be noted that defects in DNA excision repair (nucleotide excision repair and mismatch repair), and defects in DNA recombination are now thought to predispose people to various cancers, e.g., skin cancer (xeroderma pigmentosum), colon cancer (hereditary nonpolyposis colon cancer) and breast cancer (BRCA1 and BRCA2).

Maureen Hoatlin and Wade Clapp

The Fanconi Anemia Group C (FAC) Gene Product is Located in Both Nucleus and Cytoplasm of Human Cells

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The Fanconi anemia complementation group C protein (FAC) gene encodes a cytoplasmic protein of predicted Mr=63,000. The protein's function is unknown, but it has been hypothesized that it either mediates resistance to DNA crosslinking reagents or facilitates repair after exposure to such reagents. The protein also plays a permissive role in growth of CFU-GM, BFU-E and CFU-E. Purified recombinant E. coli-derived FAC antigens were used to create antisera able to identify specifically a Mr=58,000 protein in lysates from human EBV-transformed cell lines by immunoblot analysis. Subcellular fractionation of the cell lysates followed by immunoblot analysis revealed that the majority of the FAC protein was cytoplasmic, as reported previously by Yamashita and co-workers and Yousouffian; however, approximately 10% of FAC protein was reproducibly detected in nuclear fractions. These results were reproducible by two different fractionation methods, and included markers to control for contamination of nuclear fractions by cytoplasmic proteins. Moreover, confocal image analysis of human 293 cells engineered to express FAC clearly demonstrated that FAC protein is located in both cytoplasmic and nuclear compartments, consistent with data obtained from fractionation of the FA cell lines. Finally, complementation of the FAC defect using retroviral mediated gene transfer resulted in a substantial increase in nuclear FAC protein. These findings may have important implications regarding the function of the FAC protein.

In Fanconi Anemia Complementation Group C Cells, p21 (WAF1/CIP1) is Induced by Treatment With DNA Cross-linking Agents, but is Deficient in Function

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FA cells hyper-induce p53 in response to treatment with DNA crosslinking agents. We sought to determine whether p2l (WAF1/CIP1), a downstream effector of p53 checkpoint, was induced in FA cells in response to treatment with the DNA cross-linker, diepoxybutane (DEB). We found that in an FA complementation group C lymphoblastoid cell line, HSC536N, transduced with the retroviral vector, LXSN ("HX"), p2l was induced several-fold above basal levels by treatment with 1 uM DEB. Correction of the FA phenotype (hypersensitivity to DEB, and accumulation in G2/M) in HSC536N cells by transduction with the LXSN vector containing the coding region for the FA complementation group C gene, FAC ("HF"), was associated with loss of basal p2l expression, and negligible p2l induction following exposure to 1 ulM DEB. Slight p21 expression was induced in HF cells by treatment with an equitoxic (6 uM) dose of DEB, and a supra-equitoxic dose (30 uM) induced p2l levels; comparable to the basal level in HX. Similar levels of p2l induction were obtained in the HX and HF lines by treatment of cells with ionizing radiation, to which the lines are equally sensitive.

The p2l induction as described suggests that p53 functions normally in FA cells; however, the hyperinducibility of p2l in the FA line raises additional questions, which we have begun to address. In spite of the fact that the FA line readily induces p2l in response to DNA cross-linking, the cells fail to activate a G1 checkpoint (as assayed by BrdU incorporation) and they undergo re-replication, giving rise to a population of cells with greater than 4N DNA content. Both of these findings are unexpected given that p2l has been shown to mediate with G1 checkpoint and to suppress endomitosis.

To begin to investigate competence of p21 in the FAC mutant cells, we compared p2l in the HX and HF cell lines with respect to subcellular distribution and to co-immunoprecipitation with cyclin E. We found that although p2l is hyperinduced in the HX cells treated with 1 uM DEB, the majority of the protein remains in the post-nuclear supernatant. In contrast, although the level of p2l is lower in the HF line, the majority of the p2l present is contained in the nuclear fraction. Corroborating this finding, we have determined that the hyperinduction of p2l in HX cells, in response to treatment with DEB, does not result in a parallel increase in the amount of p2l binding to the cyclin E-containing complex. Both of these findings are consistent with the interpretation that in DEB-treated HX cells, p2l is induced, but is defective in translocating to the nucleus and in binding the cyclin E-cdk2 complex.

Fanconi Anemia Cells are Defective in Specific End-Joining Processes

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A high level of spontaneous chromosomal aberrations, amplified after treatment with DNA cross-linking agents, is the most important cellular feature of Fanconi anemia (FA). We previously demonstrated that the increased genomic instability is reflected at the endogenous HPRT locus by an abnormally high production of intragenic deletions (Papadopoulo et al., 1990; Laquerbe et al., 1995). To elucidate the mechanism(s) underlying the deletion proneness in FA cells, we analyzed at the sequence level the rearrangements produced within an extrachromosomal target, using the "host cell end-joining assay." The recombination substrates used allow the detection and analysis of spontaneous rearrangements and end-joining events produced during the processing of site-specific DNA double strand breaks. Moreover, these substrates permit the analysis of the products of V(D)J recombination, i.e., the formation of rearrangements with coding or signal joints.

We showed that: (i) the fidelity of V(D)J coding joints formation is significantly lower in FA-D and FA-C cells, whereas in normal cells the V(D)J machinery operates with high fidelity; (ii) blunt-ended double strand breaks are sealed with a 5–7 fold lower fidelity in FA-D and in FA-C lymphoblasts and the deletions produced are significantly larger compared to normal cells; (iii) the presence of the wild type FAC gene fully corrects the error-prone processing of DNA double strand breaks in FA-C cells. These results lead to the conclusion that at least the FAC and FAD gene products, and perhaps all FA genes, are likely to play an important role in a cellular pathway that allows rejoining of specific DNA broken ends.

FA GENE PRODUCTS: APOPTOSIS

Mutations That Inactivate the Fanconi C Gene Cause Interferon Hypersensitivity and Interferon-Dependent Cell Death

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Children with Fanconi anemia (FA) develop bone marrow failure, also known as aplastic anemia. This abnormality results in anemia (low red cell counts), thrombocytopenia (low platelet counts), and leukopenia (low white blood cell counts). The reasons children develop aplastic anemia are unknown and were therefore the subject of our research investigation. We carried out cell culture studies on bone marrow cells from mice with FA (knockout mice) of the C type and on bone marrow cells from a child with FA of the C type. We performed these studies because we had published evidence last year that the knockout mouse with FA had bone marrow cells that did not grow well in very low doses of interferon-gamma. We wanted to discover whether the cells didn't grow well because they died or simply because they didn't grow. Accordingly we measured the growth of bone marrow cells in culture dishes in the presence of both growth factors and multiple doses of interferon-gamma, then measured components of a signaling pathway (a kind of molecular bucket brigade) that causes cells to die (this molecular process is called programmed cell death, and is also known as apoptosis). We discovered that bone marrow precursor cells from FA bone marrow exposed to low doses of interferon activated the death program but normal cells did not. We then tested the idea that the FA protein (the protein made by the normal version of the Fanconi C gene) might normally suppress the death program pathway rather than the interferon signaling pathway. We found that it did not and, therefore, reasoned that the FA-C protein functions, at least in part, to slow down the interferon signaling pathway in primitive bone marrow cells and that mutations of the FA-C gene that inactivate it result in a hyperactive response and excessive programmed cell death. We believe that children with FA of the C type lose bone marrow stem cells over time and when these stem cells are exhausted (by cell death), aplastic anemia occurs.

Oxygen Dependent Cell Cycle Arrest and Mutagenesis in Fanconi Anemia Cells

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Sixty-seven FA patients and more than 1,000 controls were studied by high resolution cell cycle analysis using bivariate BrdUrd/Hoechst flow cytometry. A cell cycle disturbance, consisting of delay and arrest of cells within the S and G2 phase compartments, was found in all patients with the exception of 3 leukemic and 5 mosaic cases. However, in spite of normal cell cycle progression, the mosaic patients exhibited first cycle G2 phase fractions above 10% ($p < 0.0001$) and a small fraction of their peripheral blood mononuclear cells remains sensitive to MMC. The cell cycle defect was observed in all cell types studied (including peripheral blood mononuclear cells, umbilical cord cells, amniocytes, fetal lung fibroblasts and postnatal skin fibroblasts of various complementation groups). A very similar cell cycle pattern can be induced in normal cells by exposure to MMC or elevated oxygen tension. Since our flow cytometric assay employs BrdUrd (which is known to amplify oxygen effects), the cell cycle disturbance of FA cells is thought to reflect their oxygen sensitivity. Lowering oxygen from ambient to 5% (v/v) restores a close to normal cell cycle pattern in FA fibroblast cultures. Supplementation with excess iron induces G2 phase blockage in FA lymphoblastoid cell lines. Chelation of intracellular iron (using phenanthrolin) mitigates the cell cycle defect, suggesting ROS as a causal factor. In shuttle vector experiments (employing plasmid pZ189), both frequency and types of mutations were oxygen dependent and enhanced in FA as opposed to control cells. In plasmids recovered from FA cells grown at 20% oxygen tension, between 39 and 59% of the point mutations in the supF genes were C to T transitions, the most common type of base change induced by oxygen. These results suggest that enhanced mutagenesis causes delay and arrest of the cell cycle in FA cells grown at ambient oxygen.

Apoptosis in Fanconi Anemia-A and -C Cell Lines Treated With Mitomycin C is Associated With Redox Cycling, Not DNA Crosslinking

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Introduction

The toxicity of mitomycin C (MMC) to Fanconi anemia (FA) cells is widely assumed to be due to DNA crosslinking. MMC undergoes one electron metabolic reduction to a semiquinone that can react with DNA. However, the semiquinone can be reoxidized by molecular oxygen, with the generation of superoxide by redox cycling. This accounts for the increased toxicity of MMC to hypoxic tumor cells. Some cell lines with high expression of DT-diaphorase can reduce MMC by a two é mechanism, and formation of DNA adducts is then oxygen independent. If FA cells are defective in DNA crosslink repair, then toxicity of MMC should be dramatically increased at low oxygen, given one é reduction.

Methods

Apoptosis due to low dose MMC at either 5% or 20% O_2 was studied in normal, FA-A, FA-C, and genetically corrected FAC+C lymphoblastoid cell lines (LCLs) by flow cytometry, DNA electrophoresis and TUNEL staining.

Results

At 20% O_2 FA-A and FA-C cells showed much higher apoptosis than both normal and genetically corrected cells. At 5% O_2, apoptosis was dramatically increased in normal cells, but decreased in the FA cells, so that there was little or no difference between the groups. Chromatin condensation and large-scale DNA fragmentation were observed in the FA cells, but apoptotic bodies and nucleosomal laddering were markedly deficient in FA-A and FA-C LCLs.

Discussion

Increased apoptosis of normal cells at 5% O_2 was consistent with one é reduction of MMC. In contrast, both FA-A and FA-C cells showed decreased apoptosis at 5% O_2. This is not consistent with a DNA repair defect, but is consistent with sensitivity of FA cells to redox cycling of MMC in the presence of O_2. The qualitative abnormalities in apoptosis may help explain reports of decreased apoptosis in FA cells, based on morphological criteria.

Conclusions

FA-A and -C cell lines showed quantitatively increased and qualitatively abnormal apoptosis in response to MMC at 20% O_2, associated with redox cycling and not with DNA crosslinking.

(L to R) Filippo Rosselli, Dora Papadopoulo and Tim Rutherford

Fanconi Anemia C Acts as a Switch Between Apoptosis and Necrosis in MMC-Dependent Cell Death

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The research in our group is focused on the analysis of the role of the Fanconi anemia (FA) protein in the control of cellular homeostasis. In our previous work, we demonstrated that deregulation of apoptosis is a hallmark of the Fanconi anemia syndrome (Rosselli et al., 1995; Ridet et al., 1997). To define the role of the FA protein in the apoptotic pathway, we have extended our work to the analysis of different molecular and morphological parameters modified during the execution of the apoptotic program. In this study, we exploited the MMC pathway in FA cells from complementation group C (FAC), making this analysis informative for the mechanism involved in the sensitivity to cross-linking agents. The data obtained show that despite a higher overall level of cell death and a normal sub-G1 cell fraction for FAC compared to normal lymphoblasts, FAC cells do not display a marked DNA fragmentation indicative of apoptosis. Activation of caspases, a family of cys-

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The Fanconi Anemia Proteins, FAA and FAC, Bind and Form a Nuclear Complex

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Fanconi anemia (FA) is an autosomal recessive disorder characterized by genomic instability, developmental defects, and cancer susceptibility. Somatic cell hybrid studies have revealed five FA complementation groups (A through E). These complementation groups display similar clinical and cellular phenotypes, suggesting that the five FA genes are functionally related. The two cloned FA genes, FAA and FAC, encode orphan proteins, unrelated to each other or to other proteins in Genbank. In the current study, we demonstrate that the FAA and FAC proteins oligomerize to form a complex. Protein binding correlates with the functional activity of the FAC and FAA proteins. A patient-derived FAC protein, with a point mutation (L554P) in its carboxy terminus, fails to bind to FAA. While unbound FAA and FAC localize predominantly to the cytoplasm, the FAA/FAC complex is found in similar abundance in both cytoplasm and nucleus. Our results confirm the interrelatedness of the FA genes in a pathway and suggest the cooperation of FAA and FAC in some nuclear function, such as DNA repair, transcription, or cell cycle regulation.

FAC Acts as a Switch

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teine proteases, was described as necessary for the execution of the apoptotic program. Our data clearly demonstrated that these proteases are not active in FA cells. Indeed, the cleavage of PARP and DNA-PK proteins, targets of the caspase activity, was markedly reduced in FA cells after MMC treatment. Additionally, we show, using inhibitors of caspase activity, a central role of these proteases in MMC-dependent apoptosis in normal lymphoblasts. These caspases are not involved in FA sensitivity to MMC. Finally, the disruption of the mitochondrial transmembrane potential, which is an early event shared by apoptotic and necrotic death mechanisms, is accomplished similarly in FAC and normal cells. Altogether, our results clearly indicate that the FAC protein acts at a step of the apoptotic program preceding the activation of the caspases and after the modification of the mitochondrial transmembrane potential; this has been described in literature as a switch where cells will be pushed towards apoptosis or necrosis.

Additionally, we have analyzed these parameters using genetically manipulated FA cells overexpressing normal FAC protein. Ectopic overexpression of the FAC gene results in the inhibition of DNA fragmentation and caspase activity, while the modifications of the mitochondrial transmembrane potential are similar to those observed in both normal and FA cells. These results demonstrate a protective role of FAC protein in MMC-induced apoptosis.

Interestingly, our data demonstrate that the decrease in the mitochondrial transmembrane potential takes place independently of the FAC protein. After this step, cells lacking functional FA protein undergo a process that results in a non-apoptotic (that is, necrotic) death, responsible for the MMC hypersensitivity. In contrast, the death of cells overexpressing functional FAC protein is reduced due to the protection against apoptosis.

In conclusion, our results provide compelling evidence that normal FAC protein plays a role in the protection of MMC-induced apoptosis and demonstrate a function of the FA protein at a switch between apoptosis and necrosis.

Apoptosis Update

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Apoptosis is a type of cell death that normally takes place in humans and animals as cells age, or more dramatically, during the formation of the embryo. As an example, instead of hands with fingers, limb paddles are initially created with the tissue between the fingers subsequently regressing through apoptosis. If apoptosis does not occur normally, birth defects or cancers may result. Excessive apoptosis may result in hypoplasias of specific cell lineages during development and may be the basis of conditions such as Alzheimer's and Parkinson's disease. Apoptosis is also the principal mode of cell death following treatment of cancers with chemotherapy and is essential for the normal function of the immune system. Significant efforts have been made in the past decade to understand how apoptosis operates and whether new therapeutic modalities can target this fundamental cell process. Several components of apoptosis have been identified. A family of proteins (Bcl-2-related) function at mitochondrial and perhaps other cellular membranes and can either inhibit or promote apoptosis. A novel family of enzymes that degrade certain intracellular proteins becomes activated during apoptosis and causes important cellular damage. Apoptosis also appears to involve cell cycle dysregulation and changes in cellular oxidative stress levels. Current research is focused on understanding how these components fit together, proceeding from an initial stimulus to the demise of the cell.

Studies of Fanconi Anemia C Knockout Mice

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In the past we generated mice with Fanconi anemia group C (FACKO mice). These animals have decreased numbers of germ cells (eggs and sperm) and their bone marrow cells display hypersensitivity to interferon-gamma, an inflammatory substance made by the body during viral infections. However, they do not develop clinical anemia or cancer. We have utilized these FACKO mice to study the pathophysiology of FAC in live animals.

p53 is an important gene that is frequently deleted in many types of human cancers. Generally speaking, its normal function is to help cells avoid DNA damage and mutations. We therefore thought that generating mice that lack both p53 and fac may accelerate tumor formation and other problems in FACKO mice. We generated animals that lack both p53 and fac by cross-breeding FACKO and p53 knockout mice. Surprisingly, p53/FACKO double mutants were viable and had no developmental anomalies in addition to those seen in FACKO alone. Double mutants develop tumors faster than p53 null mice, but they are of the same spectrum (lymphomas, sarcomas). Mice that were FACKO mutant and carriers for p53 had no increase in tumor formation over pure p53 carriers. Thus the phenotypic effects of p53 loss in FACKO mice were only minor and this raised the question, whether the p53 function is important at all in the cell's response to interstrand DNA cross-links. We further investigated this by culturing cells from normal and FACKO mice and then exposing them to mitomycin C (MMC). We found that MMC exposure did not trigger increased levels of p53 in the cell. This is very different from what happens to cells after X-ray treatment or UV-light exposure and may mean that DNA cross-link repair happens during a different time in the cell cycle repair of other kinds of damage.

In human FA patients, somatic reversion has been observed at a high frequency, indicating a likely selective growth advantage of normal blood cells over FA marrow. We have used a technique called "competitive repopulation" to determine whether this growth advantage also exists in FACKO mice and how selection can be accelerated/improved. Normal mice were bone-marrow transplanted with a 1:1 mixture of Fanconi anemia group C knock-out (FACKO) and wild-type bone marrow. Analysis of peripheral blood at one month post-transplant showed neither marrow to have an early repopulation advantage. However, a further round of transplantation resulted in a >95% predominance of wild-type marrow. This indicates that selection against FACKO marrow takes place at the level of the pluripotent hematopoietic stem cell. In addition, some FACKO mice were transplanted with normal marrow cells without previous radio-ablation. Analysis at one month post-transplant showed only minimal contribution of wild-type cells in the peripheral blood. However, a single injection of mitomycin C resulted in a substantial increase in the proportion of wild-type cells in the peripheral blood. No systemic side effects were observed. Four months post-transplant, approximately 50% of the peripheral cells in the non-ablated FACKO mice were wild-type.

These results demonstrate that in vivo selection of transplanted wild-type bone-marrow cells can be achieved in FA without systemic toxicity. This strategy has potential for the improvement of gene therapy in human FA patients.

Marrow Failure in a Murine Knockout Model

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FA is a complex inherited disease that causes bone marrow failure in children. However, the specific involvement of FA genes in hematopoiesis and their relation to BM failure is still unclear. To better understand the relationship of FA gene functions to BM failure, we have generated a mouse model for FA group C and analyzed the in vivo effect of an FA-specific DNA damaging agent. Fac -/- mice were found to be highly sensitive to the FA specific DNA damaging agent which could be targeted specifically to the bone marrow to induce a progressive loss of blood cells. Thus, in vivo exposure to the FA-specific DNA damaging agent induces a phenotype remarkably similar to that seen in FA patients and suggests that the FA genes are involved in the physiologic response of hematopoietic cells to DNA damage.

Functional Interactions Between the Fanconi Anemia Group C Gene Product and Tumor Necrosis Factor-Related Death Receptors: Transgenic Mouse Models

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Fanconi anemia (FA) is a rare, inherited disorder characterized by bone marrow failure, congenital malformations, and cancer susceptibility. The FA group C gene, FAC, identified by expression cloning methods, encodes a cytoplasmic protein of unknown function. Recent evidence has suggested that FA cells may be predisposed to apoptosis. Hematopoietic progenitor cells (HPC) from FA-C patients and fac knock-out mice are hypersensitive to interferon-g (IFN-g), a molecule that can induce apoptosis through upregulation of the Fas receptor, a member of the tumor necrosis factor (TNF)-receptor family. Conversely, addition of anti-TN-Fa antibodies has been claimed to decrease the hypersensitivity of cultured FA cells to mitomycin C. We have used human FAC-overexpressing transgenic mice to study the role of FAC in the response to stimulation of TNF-related death receptors. We tested the effect of inducing apoptosis in FAC-transgenic mouse cells by incubating bone marrow cells in methylcellulose culture with IFN-g, TNFa, and/or an agonist antibody to mouse Fas. Hematopoietic progenitor cells from FAC-transgenic mice were up to 10-fold less sensitive to the cytolytic effect of Fas ligation. Our experiments implicate FAC in modulating sensitivity of HPC to Fas-mediated apoptosis. At present, very little is known about the expression levels of effector ligands such as IFN-g or the Fas ligand in FA patients. However, two groups have observed increased levels of serum TNFa in FA patients. To mimic this finding, we have mated fac-knock-out mice with transgenic mice expressing TNFa. These mice should provide further insights into the relationship between FAC and the TNF-related death receptor pathways.

Lay Summary

The first FA gene to be cloned, FAC, encodes a cytoplasmic protein. In an attempt to understand the function of this protein and how it might be involved in blood cell formation, we have created mice in which the FAC protein is overproduced in all cells of the mouse. We have found that bone marrow cells from these mice seem to be less sensitive to biological molecules that induce programmed cell death or apoptosis. These experiments suggest that the FAC protein might be involved in preventing cell death. Conversely, in patients (in whom the FAC gene product has been inactivated by mutation), the normal FAC protein may not be able to prevent inappropriate cell death. This could be a mechanism to explain bone marrow failure and some of the other manifestations of FA, giving us a better understanding of how to devise new therapies for FA patients.

Hematopoietic Differentiation of Embryonic Mesoderm is Induced by Signals from Visceral Endoderm

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The determination of cell fate during embryogenesis is governed by instructive and permissive signals emitted and received between different populations of cells in a phenomenon called embryonic induction. In nearly all vertebrate animals, embryonic blood development begins during gastrulation and results from the induction of extraembryonic (ventral) mesoderm to form hematopoietic tissue. Previous studies on developing chick embryos suggested that signals from extraembryonic endoderm were required for primitive hematopoiesis in extraembryonic mesoderm, but the nature of these signals was unknown. We have devised a novel transgenic mouse embryo explant culture system to determine whether extraembryonic (visceral) endoderm signals are required for induction of mammalian embryonic blood formation. In this assay, embryos carrying a human embryonic ß-like globin regulatory region linked to a lacZ reporter gene serve as a source of genetically marked cells in which blood formation can be followed by histochemical staining for lacZ activity. We have shown, using a tissue recombination approach, that blood formation in the mouse embryo is not autonomous to hematopoietic mesoderm alone; that is, the process requires signals from the closely apposed layer of visceral endoderm cells. Furthermore, we have shown, using conditioned medium from a visceral endoderm cell line, that the signals are diffusible and that direct cell contact between extraembryonic endoderm and mesoderm is required. These studies demonstrate that visceral endoderm signaling to hematopoietic mesoderm is conserved between mouse and chick. Finally, and most significantly, we have identified two signaling molecules that can substitute for visceral endoderm in stimulating hematopoiesis in embryonic yolk sac mesoderm. Recent studies with recombinant forms of these proteins in a variety of hematopoietic stem cell assays will be presented. Our observations may have potentially important applications to clinical medicine. The availability of a molecule that could stimulate proliferation and/or differentiation of hematopoietic stem cells (HSC) may lead to improved therapies for blood disorders such as Fanconi anemia.

CARCINOGENESIS/LEUKEMOGENESIS

Clonal Predictors of AML

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Leukemia has been reported in ~10% of patients with Fanconi Anemia (FA), and myelodysplastic syndrome (MDS) in ~5%. Patients with leukemia who do not have FA often have clonal cytogenetics. Does clonal cytogenetics in a patient with FA without leukemia mean that leukemia is inevitable? Does MDS in FA mean the same as it does in adults without FA? Are there clear predictors of leukemia in FA? MDS must be defined stringently, and includes more than just clonal cytogenetics. Among FA patients reported in the literature who developed leukemia following MDS, only one survived as long as 1.4 years. Close to 60 FA patients were reported with MDS who did not develop leukemia, and more than half survived for up to 13 years. Deaths occurred at up to 12 years, from infection, hemorrhage, or complications of bone marrow transplant.

We studied bone marrow morphology and chromosomes in two dozen FA patients seen at Mount Sinai School of Medicine and at the University of Texas Medical Branch between 1991 and 1997. Eighty percent had adequate cytogenetics, of which 6 had a clone on the first study, and 2 developed clones subsequently. One patient did develop AML. Serial studies of FA patients in the literature and in our experience demonstrated clonal fluctuation in 25% of patients, including disappearance of the clone in more than one. The adult MDS literature has provided an International Scoring System for cytogenetic classification and prognosis. Application of this to FA patients with MDS shows that the chromosome clones identified as poor risk in adults with MDS are not useful predictors in children with FA.

Clonal abnormalities are common in FA, but they tend to fluctuate and even disappear. Long-term serial studies are crucial in order to determine whether such clones have any bearing on the development of leukemia in FA. Clones in FA may merely reflect exaggerated normal clonal hematopoietic fluctuation. Markers of MDS other than clones need to be examined in FA. “MDS” in FA is not the same as MDS in non-FA patients.

Blanche Alter

Inherited Predispositions to Myeloid Leukemia

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One of the most severe and tragic complications of Fanconi anemia (FA) is the development of myelodysplasia and myeloid leukemia. These leukemias are very hard to treat because they have unfavorable biologic features and because the cells of individuals with FA tolerate conventional chemotherapy very poorly. My laboratory studies a number of other genetic conditions that are associated with an increased risk of myeloid leukemia. We have learned that these leukemias share important clinical and molecular features with each other and with the leukemias that arise in individuals with FA. These findings include a preleukemic phase, abnormalities in how a protein called Ras functions in leukemic cells, and deletions of chromosome 7. We are using a mouse model derived from studying one of the genes that is associated with an increase in the risk of childhood leukemia to try to better understand how the Ras protein works in immature blood cells and to test new treatments that are less toxic than traditional chemotherapy. We are also hunting for the gene that is lost from chromosome 7 in leukemia cells. If we are able to find it, understanding how the protein that is made by this gene works will provide important clues into the biochemical events that lead to leukemia in people with FA and other inherited predispositions, and might ultimately lead to better treatments for myeloid leukemia.

Clonal Abnormalities and Progression to Leukemia

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Fanconi anemia is due to a mutation in one of the Fanconi anemia genes. While children with Fanconi anemia are born with the disorder, they often have no blood abnormalities at birth but later develop blood abnormalities that range from anemia to aplastic anemia, myelodysplastic syndrome and acute myelocytic leukemia. Our understanding of all tumors including leukemia, as well as some pre-tumor conditions such as myelodyplasia and, in some instances, aplastic anemia, is based on the evidence that all tumors are clonal, i.e., they are derived from the mutation of a single cell that gives rise to daughter cells having the same mutation. We have developed a way of studying clonality based on the fact that each female, unlike males, has two X chromosomes but during the development of a female embryo, one becomes inactivated. By molecular biology techniques, we can trace the differences between genes derived from the mother's X chromosome as well as the father's X chromosomes. Since in adult life each female cell would have only one X chromosome with either mother's or father's X chromosome genes, normal tissue is a mixture of both cell types, while a tumor tissue would have either mother's or father's X chromosome genes but not both.

We have developed a very sensitive assay that allows determination of clonality by a transcript of the X chromosome genes suitable for all cells, even those that do not have a nucleus, such as platelets and young circulating red blood cells. The assay is sensitive and can be done on a small number of cells. The aim of our research is to determine when the blood cells in females with Fanconi anemia become clonal (derived from one blood "stem cell") and how this relates to the development of acute leukemia. We hope that with this knowledge we will be able to predict the pending development of acute leukemia before it occurs. Then, the inevitably fatal outcome of leukemia could be prevented by bone marrow transplantation, which is always more successful when done before leukemia becomes apparent.

Genomic Instability in Mice with Varying Genetic Susceptibility to Radiation Induced Leukemia

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The ability of cells to repair damaged genomes (e.g., following exposure to environmental contaminants) is critical to maintain genomic integrity. Disruption of genomic integrity is believed to be an early step in carcinogenesis. Cells from FA patients are genomically unstable and are at increased risk of accumulating genetic aberrations. Accumulation of genomic aberrations most likely contributes to the increased risk of leukemia and myelodysplasia in FA patients. Mice with varying genetic susceptibility to leukemia after radiation exposure provide opportunities to increase understanding of the propagation of cells with aberrant genomes and their participation in leukemogenesis. The genetic basis for susceptibility to radiation-induced (RI)-leukemia is poorly understood. Variation between mouse strains in ability to maintain genomic integrity may be associated with their genetic predisposition to RIL. We are exploring the role of genomic instability in susceptibility to leukemia. Specifically, we are interested in determining whether there are early markers of genomic instability in mice that develop leukemia and also increasing understanding of the propagation of cells with damaged genomes in cells of the blood system. Our data indicate that the frequency of cells with aberrant genomes varies considerably among strains. In addition, our data demonstrate that cells carrying aberrations in chromosomes that are involved in secondary leukemias in humans persist at higher frequencies in the circulation. These data will be discussed in the context of genomic instability amid leukemogenesis.

(L to R) Linda DeSpain, Leona Samson, Giovanni Pagano and Lynn Frohnmayer

Phenotype In Vivo Reversion Due to a De Novo FAA Mutation in a Fanconi Anemia Patient

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Approximately 20% of the EBV-immortalized lymphoblast cell lines established from patients with Fanconi anemia (FA) were found to be resistant to mytomycin C (MMC), in spite of the chromosomal breakage test being previously positive for MMC or diepoxybutane (DEB). In Italy, 3 out of 15 immortalized cell lines were resistant to cross-linking agents and were not analyzed for complementation group. In one of these three patients, VU393, an insertion of a single G in exon 36 of the FAA gene was detected. This mutation, which led to a frame shift and a stop codon, was inherited from both parents. However, sequence analysis of exon 36 showed also a GCTGC insertion, 20 bp downstream of the previous mutation. The GCTGC insertion created a restriction site for CfoI and restriction analysis of the family members showed that both parents did not carry this mutation.

This finding could result from an in vitro mosaicism due to a de novo mutation of one transmitted allele with selective growth in tissue culture. To determine if the de novo mutation had occurred in vivo, we analyzed DNA from a small aliquot of the blood sample obtained in 1994 from which the lymphoblastoid cell lines of both proband and his parents were established. The GCTGC insertion was found in this sample. To confirm the in vivo mutation, we analyzed DNA from a proband's blood sample obtained in 1996 and found that the mutation was still present. In addition, we compared the relative amount of the de novo mutation in blood samples obtained in 1994 and in 1996 by quantitative PCR, accounting for 25% and 33%, respectively. The results suggested an increase of the proportion, from 50 to 66%, of mosaic blood cells of this patient. Indeed, the mosaicism was not the result of a de novo mutation in parental germlines because it was not present in primary fibroblasts obtained from a biopsy of the proband's skin.

Since the de novo insertion downstream of the germline mutation restores the open reading frame, it might be that the combination of the two insertions does not abolish the activity of the FAA protein, which results altered only for 9 amino acids. A functional complementation assay using a cDNA containing both mutations is in progress. Mosaicism might be associated with a relatively mild hematological course, as reported for this patient, who has never been requiring any pharmacological treatment or transfusions. Mosaicism in FA patients has several consequences and implications for diagnosis, bone marrow transplantation and gene therapy.

Functionally Active Retroviral Vectors for Subtyping Analysis and Gene Therapy in Fanconi Anemia

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Fanconi anemia (FA) is a rare autosomal recessive disease with at least five complementation groups (A-E). Two of the FA genes (FAA and FAC) have been cloned, and mutations in these genes account for 80% of FA patients. Subtyping of FA patients is an important first step toward identifying candidates for FA gene therapy. In the current study, we analyzed a reference group of twenty-four FA patients of known subtype. Most of the patients (18/24) were confirmed as either type A or type C by immunoblot analysis with anti-FAA and anti-FAC antisera. In order to resolve the subtype of the remaining patients, we generated retroviral vectors expressing FAA and FAC for transduction of FA cell lines (pMMP-FAA and pMMP-FAC). The pMMP-FAA vector specifically complemented the abnormal phenotype of cell lines from FA(A) patients, while pMMP-FAC complemented FA(C) cells. Moreover, VSV-G pseudotyped supernatants of the vectors transduced and type-specifically improved the clonigenic survival of primary bone marrow progenitor cells from four FA patients. In summary, these functionally active retroviral vectors allow a novel, rapid method of FA subtyping and may be useful in FA gene therapy.

Bone Marrow Transplant in Fanconi Anemia: The Italian Experience

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From 1979 to April 1997, 41 Italian Fanconi anemia (FA) patients (26 males, 15 females) underwent bone marrow transplant (BMT) in ten Italian institutions. Median age at diagnosis was 6.9 (range 2.4–14.9) years. Median age at BMT was 9.6 (2.5–19.1 1) years. Median interval between diagnosis and transplant was 12 (1–118) months. At the time of transplant, 35 patients had hypo-aplastic marrow, 6 had low grade myelodysplasia. Cytogenetics (available in 33/41 patients) was normal in 30 patients; 3 had monosomy of chromosome 7. Twenty-three patients did not receive any treatment before BMT. The remaining 18 patients received androgens (9), steroids (13), cytokines (5) in various combination. Thirty-two patients were transfused before BMT with a median number of packed red cells and platelet units of 3 and 2 respectively (ranges: 1–46 and 1–37 respectively). Median pre BMT Hb was 8 g/dl (5.4–11.2), PMN 0.9 x 109/l (0–4.700), Plts 25 x 109/l (2–87).

Donors' Characteristics

Median age of the donors was 17 (5–48) years. Twenty-eight were HLA matched sibling donors (NISD), 9 were HLA matched unrelated donors (MUD). Two were HLA matched unrelated cord blood (CB). Two patients, who were transplanted from a haploidentical consanguineous donor, are excluded from the analysis.

Preparative Regimen

Patients grafted from a MSD received CY 100–200 mg/Kg (7 patients), CY 20 mg/Kg, + TAI 500–600 cGy (12 patients), CY 20 mg/Kg + fractionated TBI 500 cCy (9 patients). Patient grafted from an UD received CY 40–60 mg/Kg + TAI 500–600 cGY (7 patients) and CY 40–60 mg + fractioned TBI 500 - 1200 cGY (4 patients).

GvHD Prophylaxis

MSD transplanted patients received CSA (19 patients), CSA + MTX (8 patients), MTX alone (1 patient). UD transplanted patients received CSA + MTX (4 patients), CSA + MTX + Campath (3 patients), CSA + steroids (2 patients), CSA + Campath (1 patient) and CSA alone (1 patient).

Engraftment

A sustained PMN count > 0.5 x 109/l was reached by 85% of all patients on day + 25. A sustained platelet count > 50 x 109/l was achieved by 85% of the MDS transplanted patients on day + 42 and by 60% of UD transplanted subjects on day + 80 (p=ns).

Rejection

Rejection occurred in 2/28 (7%) of MSD and in (11%) of UD transplanted patients. All patients were retransplanted from the same donor and only one is well and alive.

Acute GvHD ≥ grade II

In the MSD group Ac.GvHD ≥ grade II involved 44% of subjects. A trend in favor of a lower risk of AGvHD was observed in patients receiving CSA + MTX (14%), compared with those receiving CSA alone (52%). In the group of UD transplants it occurred in 85% of patients.

Early Complications (within the first 100 days)

Sepsis and mucositis occurred in 41% of cases, CMV infection in 23%, hemorrhages in 20%, VOD in 17%, hemorrhagic cystitis in 13% and acute renal failure in 8%.

Survival

The probability of survival for the UD and MSD group taken together is 67% at 10 years after the graft with a plateau reached after one year. For the MSD transplants it is 81% at 5 years with a plateau achieved after 0.6 years. For the UD group the probability of survival is 25% at 4 years with a plateau reached after one year (p=0.001) The probability of survival at 5 years appears higher for patients conditioned with CY alone (86%) compared to those who received CY + TBI (77%). The group conditioned with Cy + TAI seems to have a lower probability of survival than the Cy + TBI group.

Causes of Death

Twelve patients died of transplant-related mortality at a median of 71 (10 - 293) days after BMT. In the MSD transplants, the 5 deaths were due to multi organ failure (MOF) (3), Ac GvHD (1) and ARDS (1). In the MUD group deaths occurred for CGvHD (2), AGvHD (1), MOF (1), rejection (1), hemorrhage (1), VOD (1).

Current Status of Survivors

Up to April 1997, 27/39 patients are alive with a median follow-up of 37 (5–128) months. Five out of twenty-seven patients (28%) have CGvHD. Median Karnofsky score is 90% (80–100). Median post transplant Hb is 13.6 (9–17) g/dl, PMN 3.4 (26–3) x 109/1, Plt 212 (74–358) x 109/l. No secondary tumor occurred so far.

The MSD group shows a survival curve similar to that of other international groups. Ac GvHD rate is higher than that of FA Transplant Registry (21%). In this respect Cincinnati data indicate that it is possible to reduce the incidence of Ac GvHD by adding ALG in the peritransplant.

The UD group survival is comparable to that of IBMTR (23%). GvHD represents a major problem that also has an impact on the survival rate. Future UD BMT programs in Italy will consider stronger immunosuppression (ALG ± TBI) with the aim to reduce the GvHD rate without increasing the risk of rejection.

Allogeneic Bone Marrow Transplantation In Fanconi Anemia

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FAA is a heterogeneous disorder with both genetic and phenotypic variability. Bone marrow failure is the most frequent hematological abnormality occurring typically around 5 years of age, but aplasia can appear later. Clonal abnormalities including a high frequency of monosomy 7 and duplications involving 11q can be observed on marrow cytogenetic analysis as a sign of transformation to myelodysplastic syndrome or acute myeloblastic leukemia. Without curative treatment, spontaneous survival is poor, death occurring during the second decade of life from aplastic anemia, leukemia or cancer.

HLA Identical Sibling Transplants Results

Bone marrow transplantation is the only treatment that restores definitely normal hematopoiesis. Cyclophosphamide (Cy), which is used for the conditioning of patients with idiopathic aplastic anemia at the total dose of 200 mg/kg, has been proven to be too toxic, leading to a high rate of transplant-related mortality. For this reason, the conditioning regimen for bone marrow transplantation was modified by our team in 1980; it includes Cyclophosphamide 20 mg/kg given IV over 4 days and a 5 Gy thoraco-abdominal irradiation, followed by Cyclosporin A alone for prevention of GVH. In the study performed by our center, including 45 patients who had received an HLA identical sibling bone marrow or a cord blood transplant, the 5-year actuarial disease-free survival was 75.6%. Study of chimerism showed that all engrafted patients were of donor type with no residual host cells. This confirms that the decrease of the dose of immunosuppression was sufficient to induce long-term tolerance and absence of autologous reconstitution. In 159 patients collected by the European Registry of the EBMT, the actuarial survival was 68%. The estimate of developing a solid tumor by 20 years after transplantation was 42% among patients with Fanconi anemia. They were all head and neck squamous carcinoma. The fact that, in Fanconi anemia patients, only solid tumors and no lympho myeloproliferative malignancies were observed post-transplant is consistent with the fact that transplantation provided patients with a normal hematapoietic system, but did not affect the congenital defect in other tissues.

Alternative Donor Transplants

In the EBMT registry, we have analyzed patients transplanted with an HLA identical unrelated donor (n=36), a mismatched related donor (n=11) or an unrelated mismatched donor (n=9). Transplantation consisted of bone marrow in 47 cases and cord blood in 9 cases with a median cell dose of 2.5x108/kg. Conditioning consisted of low dose Cyclophosphamide associated with TBI (n=18) or TAI (n=35). Ex vivo T cell depletion was used in 10 cases. ATG or monoclonal antibody was used in 45 patients. Engraftment was observed in 42 cases, 13 patients had a secondary marrow failure, 9 patients received a second BMT, 2 are currently alive. The probability of developing GVH grade II-IV was 62%. Chronic GVH was observed in 8/25 patients at risk. The two-year survival was 30%. Factors associated with a better outcome in univariate analysis were: T cell depletion (p=0.01), short interval from diagnosis to BMT (p=0.02), HLA identical unrelated versus a mismatched donor (p=0.026), bone marrow versus cord blood (p=0.035), and young age (p=0.048). In a Cox model, T cell depletion and short interval between diagnosis and BMT remained significant factors associated with survival.

New Strategies For Therapy

1. Cord blood transplant

In Eurocord, 16 patients received a cord blood transplant for Fanconi anemia, 8 were transplanted with a related donor, 5 are currently alive and well. Eight received an unrelated CBT and one patient is currently alive.

2. Gene therapy

Autologous transplant has been discussed but seems of very limited value in Fanconi anemia where the few CD34+ cells isolated from G-CSF mobilized HSC from the blood do not grow in long-term culture and are unlikely to give a short- or long-term engraftment. Collection of cord blood at birth or of peripheral blood CD34+ cells can be performed at an early phase of the disease for gene transfer. The recent localization of two new genes and the demonstration in-vitro that transfected cells had a selective growth advantage on Fanconi anemia cells has increased the interest for design of gene therapy protocols in Fanconi anemia. Several questions remain unsolved, including the integration of the gene in primitive hematopoietic stem cells, the level of integration necessary for the correction of the disease, the long-term expression of the transfected gene, the selective growth advantage of transfected cells, and the function of the FA proteins.

Overview of Fanconi Anemia Gene Therapy and Update on the FA-C Experimental Trial

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Fanconi anemia (FA) is an autosomal recessive disorder that leads to aplastic anemia. For patients lacking a histocompatible stem cell donor, therapy is limited to supportive care. Mutations in a gene called FAC account for 10–15% of FA cases. Cells from FA patients are abnormally sensitive to DNA-damaging agents such as mitomycin C. Transfection of normal FAC into mutant cells corrects this hypersensitivity and improves their viability in vitro. Three FA patients, each bearing a different FAC mutation (exon 1, exon 14, intron 4), were entered into a clinical trial of gene transduction aimed at correction of the hematopoietic cell defect. Each patient received three or four cycles of gene transfer, each consisting of one or two infusions of autologous hematopoietic progenitor cells that had been transduced ex vivo with a retroviral vector carrying the normal FAC gene. The FAC transgene was demonstrated in transduced CD34-enriched progenitor cells. Following infusion, FAC was also present transiently 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. Transduction of the normal FAC gene into hematopoietic cells from patients with FAC mutations can improve the pathologic process affecting FA hematopoiesis. However, despite the in vitro selective advantage resulting from FAC gene transfer, we did not observe long-term hematopoietic reconstitution with gene-corrected clones. Based upon these results, we have modified our protocol in two ways. First, we intend to compare transduction of bone marrow versus peripheral blood as a source of hematopoietic progenitor and stem cell targets. Second, we will examine whether autologous bone marrow cells transduced with the FAC transgene can be used to reconstitute FA-C patients who have developed graft failure following allogeneic stem cell transplantation from an unrelated donor. In the latter instance, additional selective pressure for FAC gene-modified cells may exist because the recipient will have received both immune suppression and chemotherapy conditioning.

Lay Summary

We have been involved in the development of experimental trials of gene therapy for Fanconi anemia. Studies in our laboratory have suggested that Fanconi anemia may be a good candidate disease for gene therapy. We have placed the normal Fanconi anemia type C gene into a retroviral vector and introduced the gene into cells derived from Fanconi anemia type C patients. In the laboratory, when we have compared cells lines and bone marrow cells from Fanconi anemia patients before and after this procedure, we see a return towards normal of cell growth, resistance to the chemical agents that harm Fanconi anemia cells, and a more normal appearance of the cells' chromosomes. The purpose of our research protocol is to test whether we can safely introduce the normal Fanconi anemia type C gene into stem cells of patients with this disease. Stem cells are the cells in the bone marrow and blood that give rise to the white cells, platelets, and red cells. We will treat blood or bone marrow cells from patients with Fanconi anemia type C using the vector containing the Fanconi anemia gene in a test tube and then return these cells to patients. If the cells are genetically altered, we expect to be able to detect the normal gene in blood and bone marrow cells afterwards. We hope that cells that contain the Fanconi anemia gene will grow well in the bone marrow, and that we will be able to detect normal stem cells in special tissue culture studies.

Jerome Lo Ten Foe, Johnson Liu, and Christopher Walsh

Gene Therapy for Fanconi Anemia

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The transfer of correct FA genes into the bone marrow cells isolated from FA patients is a potentially powerful method for the treatment of this most devastating aspect of the disease. FA is a prime disease to explore this mode of therapy. Why? For the following reasons: two FA genes, FAA and FAC, have been isolated. Viral vectors that carry these corrected FA genes to target cells such as bone marrow, cord blood and peripheral blood cells are constantly being improved and new vectors developed. Purification methods for the isolation of target cells (hematopoietic stem cells) for such vectors have been improved. Basic understanding of the interaction between these vectors and the target cells has allowed us to try a variety of different strategies to insert the correct FA genes into cells. Most importantly, the transfer of correct FA genes into mutant hematopoietic cells confers a growth advantage to corrected cells and can be easily performed in the laboratory. This improved hematopoietic cell growth following gene transfer should allow reconstitution of blood cell formation in FA patients. In addition, we can now diagnose unclassified patients using gene transfer. Our aim at the UNC Gene Therapy Center is to improve upon current methods for gene transfer and develop new ones for the future.

Vicki Athens, Ralf Dietrich, Lynn Welfare and Hans Joenje compare notes on mosaicism.

Spontaneous Lymphocyte Mosaicism in Fanconi Anemia: an Example of Natural Gene Therapy?

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Mosaicism refers to “a condition in which an individual has two or more cell lines of different genetic constitution.” Mosaicism thus results from alterations in genes occurring during an individual’s lifetime. It should be realized that the DNA contained in an organism is not 100% stable. Alterations may originate from wrongly repaired DNA damage or from errors in the DNA copying process that precedes cell division. Such types of events have contributed to the “mutational load” of the human species, causing all individuals to carry mutations in at least some genes. Only individuals who carry two mutated copies of an FA gene suffer from FA. In FA patients recognized as being “mosaic,” two types of lymphocytes are observed, one with the typical MMC hypersensitivity (FA-like) and one with normal sensitivity (non-FA like).

Two types of observation suggest an FA patient to be mosaic:

1. a lymphoblastoid cell line (derived from a B lymphocyte) has a normal (non-FA like) MMC sensitivity;

2. in the standard diagnostic chromosomal breakage test (carried out with T lymphocytes) a significant proportion of the cells behaves as non-FA.

The occurrence of normal cells in an FA patient may be explained by secondary DNA alterations in the mutated FA genes giving rise to at least one normal copy of the gene. Generation of a normal FA gene might result from “mitotic recombination” in compound heterozygous patients or from a secondary mutation that somehow nullifies the primary pathogenic mutation.

We are only just beginning to understand the molecular basis of mosaicism in FA patients and are still far from knowing its precise clinical implications. In some patients, but not in others, the level of mosaicism has reached a point where the standard chromosomal breakage test would no longer be positive for FA; such cases seem to be associated with relatively mild hematological symptoms. On the other hand, clear examples exist of non-mosaic (100% FA-like) patients who have only mild symptoms despite a relatively old age. Such cases might represent relatively subtle changes of the affected FA gene (“mild mutations”). Much research remains to be done to better understand the clinical significance of mosaicism in FA.

Unrelated Stem Cell Transplant for Fanconi Anemia: Multicenter Report of Forty-Nine Cases

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Most patients with Fanconi anemia (FA) will develop hematological abnormalities (median age of onset 7 years), which often progress to bone marrow failure, myelodysplastic syndrome (MDS) or acute myelogenous leukemia (AML). While bone marrow transplant (BMT) is the only treatment with curative potential, few patients with FA have suitable HLA-matched related donors and until recently few patients have been treated successfully with unrelated donor (URD) BMT. We have analyzed the outcome of URD BMT for patients with FA from transplant centers in the U.S. and Europe and present data on 49 cases. Median patient age was 12 years (range 2–48 years); 30 were male. Thirty-one cases were aplastic at BMT, 12 had MDS and 6 frank AML. Thirty-four percent had a clonal chromosomal abnormality prior to BMT. Four received cord blood grafts and the remainder bone marrow. Stem cells had a single HLA mismatch at A, B or DRB1 in 20. Most recipients received conditioning therapy with TBI (69%) or TLI (24%) and reduced dose cyclophosphamide (20 or 40 mg/kg). Grafts were T-depleted in 37%; the remainder received GVHD prophylaxis with cyclosporin and corticosteroids ± other (56%); methotrexate ± other (20%) or other drug combinations (24%). Median follow-up is two years (range 0.1–9 years). Overall, 75% achieved hematopoietic engraftment (ANC > 0.5 x 109/L) by 42 days post-BMT; 22% received a second stem cell infusion for primary or secondary graft failure. GVHD grades II-IV occurred in 52% receiving non T-depleted and 29% receiving T-depleted marrow (p=0.12) and in 24% receiving HLA-matched and 67% HLA-mismatched marrow (p=0.02). Overall disease-free survival was 38% (95% CI 24–52%); survival in those age < 5 years was 69% (95% CI 32–100%) (p=ns). Survival was 43% (95% CI 23–63%) in those receiving HLA-matched marrow and 32% (95% CI 10–54%) in mismatch recipients. In univariate and multiple regression analysis, stratified by center, the only significant variable influencing survival was chemotherapy dose; patients receiving 40 mg/kg of cyclophosphamide were more likely to survive than those receiving 20 or 30 mg/kg (p=0.03; relative risk 0.36). These data indicate that URD BMT should be considered as a therapeutic option for patients with FA. Graft failure remains a major problem, with better survival in patients receiving a higher dose of chemotherapy. These data form the basis for an ongoing conditioning therapy dose escalation study.

(L to R) Lynn Sablosky, Karen Siebenthal and April Benton enjoy the FA Symposium.

Engraftment and Proliferation of Retroviral-Transduced Human Hematopoietic Progenitor/Stem Cells in Fetal Sheep

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The study of human primitive hematopoietic cells and evaluation of gene transfer protocols require the use of assays that permit in vivo analysis of transduced cells. Despite the many useful features of xenograft transplantation in fetal sheep, it remains unclear whether transduced human hematopoietic progenitors/stem cells can engraft and proliferate in sheep/human chimeras. We transduced purified human CD34+ bone marrow, or cord blood cells with a recombinant retrovirus (vMFGFAC) by coculture or using supernatant transductions on fibronectin fragments and transplanted the transduced cells via intraperitoneal injection into day 45–50 gestation sheep fetuses. In order to determine whether transduced human cells engraft in fetal sheep, three fetuses were sacrificed at day 130–140 gestation (80–95 days after transplantation). Two of three fetuses analyzed contained both human-specific ß-globin and proviral sequences in the peripheral blood, bone marrow and spleen at autopsy, indicating engraftment of transduced human hematopoietic cells. In order to evaluate the proliferation potential of the transduced cells, nine lambs are being analyzed sequentially. Bone marrow and peripheral blood samplings from one sheep have been obtained from 3 to 18 months after birth. Fluorescence activated cell sorting was performed to isolate human T cells (CD3+) and CD34+ cells. In addition, human progenitors were cultured from bone marrow cells. Integration of the retrovirus in human cells was demonstrated by polymerase chain reaction of proviral sequences in both human T cells and in individual progenitors. Inverse PCR on individual progenitors was also performed to analyze the proliferation of individual primitive progenitor cells as a function of time. Three unique integration sites, indicating the proliferation of three distinct progenitor/stem cells, were detected at times ranging from 6 to at least 18 months of age. One integrant was detected in progenitors from 4 samplings over 10 months (6 to 16 months) indicating long-lived proliferation of a primitive cell. These data demonstrate that transduced human hematopoietic progenitor cells are capable of engraftment in fetal sheep. The data also demonstrate that individual human hematopoietic progenitor/stem cells have the capacity to proliferate in vivo in this model for prolonged periods.

Mutation Screening of the FAA Gene

continued from page 4

analysis. These techniques have revealed a heterogeneous mutational spectrum in the FAA gene ranging from single base changes leading to aberrant splicing, amino acid substitution or premature termination of translation, small genomic deletions leading to a shift in the reading frame, through to larger genomic deletions that remove up to 40% of the transcript (as well as causing a frameshift). The broad spectrum of mutations observed suggests a need for a wide-ranging approach to mutation screening, since each of the above techniques is likely to detect only a subset of mutations, especially in compound heterozygotes.

We have investigated the origin of the largest genomic deletion in the FAA gene for which the extent of the deletion is known. Comparative sequencing of breakpoint PCR products from patients carrying the deletion with control samples has revealed the presence of Alu-like elements in introns 11 and 31 that appear to have undergone non-homologous recombination with the effect of deleting about 40 kb from the genome (up to half of the FAA genomic sequence). This mutation is found in approximately 65% of South African Afrikaner FA patients. We have used fluorescent dosage analysis of this and other characterized deletions to detect carriers, and have performed two prenatal diagnoses in pregnancies at risk of Fanconi anemia.

Complementation Studies in FA

continued from page3

distinct groups (A-H) have been found among fewer than 30 patients investigated from Germany and The Netherlands. Only the genes for groups A and C have been identified, which means that in patients belonging to these groups, the disease-causing mutations can be identified. The groups F-H are currently represented by single patients only, whereas multiple patients have been assigned to groups A-E.

Mutations in FA-A and FA-C account for an estimated 75% of all FA patients worldwide (A: 65%, C: 10%), but these frequencies may widely differ depending on the population (e.g., 80% type C in people of Ashkenazi Jewish ancestry; close to 100% type A in Afrikaans-speaking South Africans and Italians). For patients who are not in the A or C groups, complementation analysis is currently the only way to assess their subtype. Before the disease-causing mutations can be assessed in these patients, the corresponding genes must be identified.

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