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Feature Articles

Fanconi Anaemia Research: Current Status and Prospects

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Fanconi anaemia (FA) is an autosomal recessive disease featuring diverse clinical symptoms in addition to chromosomal instability and hypersensitivity to crosslinking agents. The much increased risk of FA patients developing leukaemia and squamous cell carcinomas makes FA an important model disease for cancer predisposition. Studies documenting the characteristics of FA cells and their response to environmental toxins have failed thus far to disclose the basic cellular process that is primarily disturbed in FA cells. Complementation analysis suggests that mutations in at least four different genes can cause FA (complementation groups FA-A to FA-D). The cDNA for FA-C has been cloned and found to encode a novel protein that localises to the cytoplasmic compartment of cells. Even though the protein's function is still unknown at present, research has now reached the point from where rapid progress to a detailed understanding of this syndrome may be foreseen.

Key words: chromosomal instability, leukaemia, cancer, predisposition, bone marrow failure, haematopoiesis, crosslinkers
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INTRODUCTION

SEVERAL REVIEWS have been published discussing clinical and basic aspects of Fanconi anaemia (FA) [1–7]. This paper, apart from giving an introductory overview, particularly summarises recent developments emerging from the genetic approach to elucidate this disease.

FA is a rare autosomal recessive disorder with an estimated frequency of homozygotes in the order of 1–3 per million. FA is observed worldwide, and is not restricted to any particular human population. FA is known as a cancer-prone chromosomal instability syndrome, since cultured cells from FA patients exhibit elevated levels of 'spontaneous' chromosomal aberrations and a hypersensitivity to crosslinking agents. FA thus represents a challenge to elucidate a specific pathway controlling genomic stabilisation in mammalian cells: the 'FA pathway of anti-oncogenesis'.

CLINICAL SYMPTOMS

Manifestation of the disease is extremely pleomorphic and may include congenital malformations, abnormalities of skin pigmentation and skeletal (radius and thumb dysplasia) and renal anomalies. Because of growth retardation, the short stature of the affected siblings is frequently obvious, but in some families affected siblings appear virtually normal and have normal growth. In those cases, the FA diagnosis is based on spontaneous and crosslinker-induced chromosomal instability in lymphocyte cultures.

HAEMATOLOGICAL SYMPTOMS AND CANCER IN FA

Pancytopenia is an important clinical hallmark of FA, which typically develops between 5 and 10 years of age, and usually starts with thrombocytopenia, followed by granulocytopenia and anaemia, which develop more slowly. In FA patients, the predominant cancer is acute myeloblastic leukaemia (15 000-fold increased risk), although a variety of solid tumours is also observed [1, 6, 8]. The cause of bone marrow failure is unclear. *In vitro* colony assays show a severe depletion of haematopoietic colonies [9]. Treatment of bone marrow cells with the antisense oligodeoxynucleotide complementary to bases −4 to +14 of mRNA of the FAC gene inhibited clonal growth of erythroid and granulocyte–macrophage progenitor cells, indicating that this FAC gene product plays a role in regulating growth, differentiation and survival of normal haematopoietic progenitor cells [10]. Abnormalities of cytokine production have been described such as an increased production of TNF α and decreased production of IL6; these abnormalities are unlikely to be the cause of aplastic anaemia but rather the consequence of the genetic defect [11, 12]. Initially, the patients respond favourably to androgens or to G or GM-CSF but, progressively

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pancytopenia returns, the patients become refractory to transfusions and die of severe aplastic anaemia or acute myeloid leukaemia. In a recent study of the international Fanconi Anaemia registry [13], the actuarial risk of developing clonal cytogenetic abnormalities during bone marrow failure was 67% (47–87%) by 30 years of age. Risk was higher in patients with, than in those without, a prior clonal cytogenetic abnormality (35% versus 3%). The frequency of developing leukaemia in FA patients is higher than in idiopathic aplastic anaemia treated by immunosuppression, where an actuarial risk of developing myelodysplastic syndrome or acute myeloid leukaemia is 30% [14]. The physiopathogenic link between these two preleukaemic disorders is not yet known. The best treatment of FA is allogeneic bone marrow transplantation with a healthy HLA identical sibling using bone marrow or cord blood collected at birth [14]. It has been recognised that the *in vitro* sensitivity to alkylating agents resulted in a poor tolerance to high dose cyclophosphamide used for conditioning patients with severe aplastic anaemia. A modified protocol using low dose cyclophosphamide and lymphoid irradiation gives 80–90% long term survival. In patients treated with allogeneic bone marrow transplantation, an increased risk of developing head and neck squamous carcinoma has been observed with or without using irradiation for conditioning. An increased risk of developing various cancers has also been observed in non-transplanted patients. The recent discovery of the gene responsible for group FA-C (see below) may give some insights into the cause of leukaemia and cancer in this group of patients.

SYMPTOMS IN HETEROZYGOTES

The frequency of heterozygotes is estimated at 1 per 300, based on the assumption that the disease is caused by a single gene defect. If genetic heterogeneity is taken into account (see below), the heterozygote frequency may be as high as 1%. There is evidence that heterozygotes tend to have some mild manifestations of the disease, both in terms of clinical symptoms (skeletal proportions, haematological parameters [15]; clastogen-sensitivity [16] and cancer risk [17, 18]. The increased cancer risk in FA heterozygotes was not confirmed in a later study by the same author [19, 20]. This question is unlikely to be resolved until a much larger data set of cancer risks in obligate heterozygotes is analysed. Such data could be expanded if a reliable laboratory test for heterozygotes were available, but this will depend on the cloning of the genes for the major complementation groups.

CELLULAR CHARACTERISTICS

The most striking characteristic of FA at the cellular level is an increased spontaneous chromosomal instability, the damage being mainly of the chromatid-type, including chromatid gaps, breaks and interchanges. The instability includes cytogenetic breakpoints frequently involved in acute myelogenous leukaemia [21]. A second hallmark of FA cells is their dramatically increased sensitivity to the clastogenic (chromosome-breaking) and cytostatic effects of crosslinking agents such as mitomycin C (MMC), diepoxybutane (DEB), 8-methoxypsoralen plus long-wave UV, cyclophosphamide, and cisplatinum.

ABERRANT PROCESSES IN FA CELLS

Attempts to link the abnormal characteristics in FA to cellular processes have mainly focused on the following, presumably interrelated, processes: DNA repair, response to oxygen, cell cycle kinetics, and cytokine production and response.

DNA repair

Because of the excessive chromosomal damage typically observed in FA cells, FA has been considered a putative DNA repair disorder [22]. The rather specific hypersensitivity of FA cells to crosslinking agents led Sasaki [23] to propose that FA cells might be deficient in the repair of DNA interstrand crosslinks. The crosslink repair deficiency hypothesis is presently controversial, as several laboratories have generated conflicting results, which may have resulted from the use of cell lines belonging to different complementation groups.

Abnormal response to oxygen

Since spontaneous chromosomal breakage was shown to be positively correlated with the atmospheric oxygen concentration in lymphocyte cultures (range 3–45% O_2), it has been proposed that the primary defect in FA might be somewhere in the complex system of defence against oxygen toxicity [24–26]. This system consists of components that prevent the excessive formation of potentially damaging reactive intermediates as well as components that repair such damage. Normal cells chronically exposed to hyperoxic conditions are hypomutable [27], like FA cells kept under normal conditions [28]. Hoehn and associates [29] have proposed that the FA mutation causes a defect in cell cycle traversion resulting in G2 phase prolongation as well as complete G2 phase arrest and a reduced growth at ambient oxygen levels. Near-normal growth and cloning efficiencies could be restored in FA fibroblasts by reducing the ambient O_2 levels from 21 to 5% [29]. Even though an abnormal response to oxygen is related to the FA defect, the finding that oxygen hypersensitivity (but not MMC hypersensitivity) is lost after SV-40 transformation of FA fibroblasts suggests that oxygen sensitivity may be an indirect manifestation of mutated FA genes [30].

Other cellular aberrations

An intriguing observation, that might be critically related to the bone marrow defect in FA, is that FA cells seem to have an abnormality in cytokine production [11, 31–33], which may be related to abnormalities in the production of oxygen radicals by FA leukocytes [34]. Bagby and colleagues [35] reported abnormal patterns of cytokine production in FA fibroblasts, but found discordant results with patients from the same sibship, and concluded that the abnormalities were probably not directly related to the FA defect.

In summary, the phenotypic manifestations of FA are diverse, both at the clinical and cellular levels. It remains to be established which of the abnormal characteristics of FA cells are primary and which are secondary manifestations of the FA defect(s). Part of the diversity in characteristics may be related to the existence of genetic subtypes.

AT LEAST FOUR FA GENES

Complementation analysis of cell fusion hybrids revealed the existence of at least four complementation groups in seven lymphoblastoid cell lines from unrelated FA patients [36]. The analysis depended on the introduction of selection markers in the fusion partners to allow positive selection of heterokaryons. This was accomplished through transfection with plasmids conferring either hygromycin or G418 resistance. Since transfection efficiencies are typically low in human lymphoblasts, this procedure is not suitable for large-scale assignment of unclassified FA patients to a complementation group. However, genetic variants marked with neomycin resistance and sensitivity to

medium containing hypoxanthine, aminopterin and thymidine have recently been prepared from a panel of cell lines representing each of the known complementation groups (Joenje and associates, unpublished and [37]). This panel can now be used to carry out genetic classification of any FA patient from whom a well-growing mitomycin C-sensitive lymphoblast line can be derived. The European consortium on FA research, EUFAR, is presently carrying out a large-scale complementation analysis to assess the number of major FA genes in European countries, which may lead to the identification of new complementation groups.

THE FIRST FA GENE CLONED: *FAC*

Several strategies are currently being used to clone the genes involved in FA: positional cloning through linkage analysis [38], interspecies complementation by transfection with genomic DNA [39] and expression cloning (see [3] for a more comprehensive discussion). The latter method has been successful for *FAC*. Strathdee and associates [40] isolated the "FA group C-complementing gene" (*FAC*, see [41] for nomenclature) by transfecting a cDNA library, cloned in an EBV-based shuttle vector, into the FA-C lymphoblast line HSC536. The gene appeared to be altered in the original cell line HSC536 by a T to C transition at nucleotide position 1913 that changes codon 554 in the maternal allele from a leucine to a proline (L554P); the paternal allele was not expressed in HSC536 cells. *FAC* has been mapped by *in situ* hybridisation to chromosome 9q22.3, in close proximity to the xeroderma pigmentosum complementation group A gene, *XPA*. A polymorphism within *FAC* has been used to place it within a 5 cM interval on the genetic map, flanked by the microsatellite loci D9S196 and D9S197 proximally, and D9S176 distally [42]. This interval also contains the genes for Gorlin's syndrome and multiple selfhealing squamous epitheliomata, which have not yet been identified. Linkage analysis in FA families with polymorphic markers surrounding *FAC* has shown that the disease was linked to this locus in only approximately 8% of unclassified FA families [42].

FAC: GENE STRUCTURE AND MUTATIONS

The genomic structure of the coding region of the *FAC* gene was resolved by vectorette PCR analysis [43]. The 1674 nucleotide coding sequence is highly interrupted, being composed of 14 exons which range in size from 53 to 204 bp. All the exon donor and acceptor splice sites fit well with consensus sequences. The 5′ and 3′ untranslated regions of *FAC* have not yet been fully characterised. FAC transcripts which lack exon 13 have been detected in both FA patient and control RNA [44]. Removal of exon 13 would not cause a frameshift of the coding sequence, and may, therefore, generate a protein with a slightly altered function. Intron sequences have been used to design PCRs to amplify the 14 exons [43], which permits screening and characterisation of *FAC* mutations.

The *FAC* gene has been screened for mutations in FA patients by reverse transcription of FAC transcripts followed by chemical cleavage of PCR amplified cDNA [44–46] and also by single-strand conformational polymorphism (SSCP) analysis of the individual exons [47]. A total of six pathogenic mutations have been identified (see Figure 1), of which four would result in premature termination of translation of the FAC polypeptide. IVS4+4A>T results in loss of exon 4 from the FAC transcript [45], and L554P has been shown to be associated with loss of FAC function [48]. The IVS4+4A>T mutation accounts for the majority of FA in Ashkenazi Jewish individuals [45, 47], with a carrier frequency of 1/157 in persons of Jewish ancestry [49]. Two other coding sequence mutants (G139E and S26F) have been shown to be polymorphisms since they do not segregate with the FA phenotype, whilst the pathogenic status of the D195V mutation remains to be established [47]. The frequency of pathogenic mutations can be used to estimate the proportion of FA patients who belong to group C. Combining the chemical cleavage data of Whitney and associates [45] with those of Gibson and associates ([44] and manuscript in preparation) produces a figure of 5/47 (10.6%) of FA-C patients, whereas Verlander and colleagues [47] detected mutations in 14.4% (cf. [42]). This higher figure may be the result of the higher proportion of patients with Jewish ancestry in that sample. The number of FA patients with specific FA mutations is still too limited to allow correlations of genotype with clinical phenotype, except perhaps for the common IVS4+4A>T mutation, which is associated with a severe phenotype of multiple congenital malformations and early onset of haematological disease [47]. In cases where a pathogenic mutation has been identified in the *FAC* gene, the information may be used for early prenatal diagnosis of FA by DNA analysis [46]. The murine homologue (Fac) of the human FAC cDNA has been cloned by Wevrick and associates [50]. The mouse Fac protein shares 79% amino acid sequence similarity. Cross-hybridization sequences exist in other mammals, chicken and *Drosophila* [50]. The mouse gene maps to chromosome 13, in close proximity to the anaemic mouse mutation flexed-tail. There is no evidence, however, that flexed-tail is the mouse homologue of *FAC*, even though its candidacy has not been excluded [51].

STUDIES ON THE FAC POLYPEPTIDE

FAC is ubiquitously expressed, albeit not abundantly, except for early developing bone tissue [5]. The predicted FAC gene product is a novel 558-amino acid protein with no known functional motifs. In spite of the relatively low level of sequence identity between human and mouse FAC, the mouse Fac cDNA is still able to fully complement MMC sensitivity of human FA-C cells, indicating that a variety of coding sequence changes could be tolerated by FAC without affecting its protein's function. Most disease-associated mutations appear to cause truncated proteins, except the original missense mutation [40], which causes a leucine to proline substitution at the C-terminal region of the protein. This indicates that at least the C-terminus is important for activity. Targeted mutagenesis studies will be required to obtain a more detailed delineation of the regions that play a role in the protein's biological function.

In the absence of any clue to the function of the FAC gene product, antibodies are a vital tool to study the protein's role in the cell. Yamashita and associates [52] recently succeeded in preparing rabbit antibodies against the carboxy-terminal half of the FAC polypeptide fused to glutathione S-transferase. The antiserum was able to recognise and precipitate a protein from cell homogenates with the predicted molecular mass of about 60 kDa, whereas in FA-C patients truncated proteins were detected, as expected from the mutations identified. Interestingly, two more proteins, one of 50 and one of 150 kDa, were also recognised by the antibody. The 50 kDa product most likely represents a FAC mRNA splice variant, whereas the 150 kDa protein is probably the product of a separate gene encoding a protein with homology to FAC, and may represent a gene from one of the other FA complementation groups. Cell fractionation studies, using lymphoblastoid cell lines, indicated that all three proteins were localised in the cytoplasmic fraction.

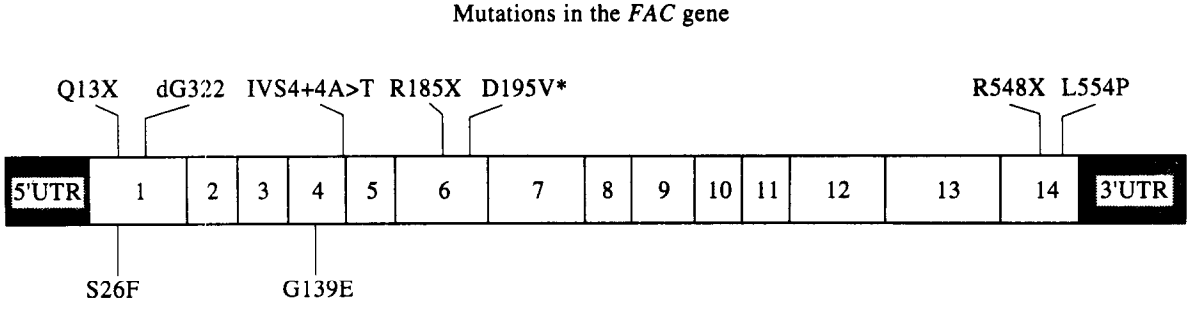

Figure 1. Coding sequence changes detected in the 14 exons of the *FAC* gene. Mutations shown above the gene are pathogenic, and those below are polymorphisms. *, pathogenic status unknown; d, deletion; UTR, untranslated region.

This localisation was confirmed with N-terminus epitope-tagged FAC expressed from an EBV-based shuttle vector transfected into FA-C lymphoblasts. The tagged protein, which apparently complemented the crosslinker hypersensitivity of the transfected cells, was shown by immunofluorescence to localise exclusively to the cytoplasmic compartment. Similar results were recently reported by Youssoufian [53]. This result seems to exclude a role for FAC and FAC-related proteins as participants in a DNA repair or replication complex. However, it is not yet known whether genotoxic conditions, particularly those resulting from crosslinkers or oxidative stress, would trigger the translocation of these proteins to the nucleus where they could then become directly engaged in DNA repair.

NEW THERAPEUTIC OPTIONS: GENE THERAPY

Now that the FAC cDNA has been cloned, gene therapy has become a therapeutic option for the majority (>75%) of group-C patients for whom there is no suitable HLA-matched bone marrow donor available. Gene therapy may be curative for the immediate life-threatening symptoms related to the bone marrow dysfunction, i.e. aplastic anaemia and predisposition to leukaemia. The scenario would be to mobilise and collect bone marrow stem cells from peripheral blood, carry out *ex vivo* gene transfer using a recombinant retroviral [54] or adenovirus-associated expression vector [55] containing the wildtype coding sequence of *FAC*, and reinfuse the corrected cells into the patient. Since the primary defect in FA bone marrow primarily affects the pluripotent stem cell, the likelihood of success for FA patients may be higher than in severe combined immune deficiency, where primarily the lymphocyte lineage rather than the pluripotent stem cell is affected. In June 1994 Dr J.M. Liu and co-workers at the National Heart, Lung and Blood Institute, Bethesda, Maryland, U.S.A., obtained approval from the Recombinant DNA Advisory Committee for a gene therapy protocol involving FA-C patients; the protocol is now awaiting approval from the Food and Drug Administration (FDA) (J.M. Liu, personal communication). A major problem for the successful application of this novel therapy in FA patients may be the lack of sufficient mobilisable stem cells in patients who are in aplastic phase. In order to circumvent this problem, it may be necessary to collect stem cells before patients become aplastic. Given the relatively low prevalence of FA-C, only a minority of FA patients will be eligible for gene therapy. However, if the procedure worked for group C patients without unaccepable complications, it could be adopted for patients of other complementation groups, once the corresponding genes had been isolated.

ANIMAL MODELS

The technique of generating animals in which a specific gene has been inactivated by targeted mutagenesis has proved to be a valuable tool to study gene functions *in vivo*. Such a model system for FA-C is presently being developed in several laboratories. If the clinical symptoms of FA are expressed in *Fac* knock-out animals, a new area of research activity will be opened up: not only will the animal model allow the study of fundamental processes controlled by the FA genes, including developmental, haematological and cancer-related aspects, but it may prove to be invaluable for the development of new therapeutic strategies.

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