Dyskeratosis Congenita Fibroblasts Are Abnormal and Have Unbalanced Chromosomal Rearrangements

By Inderjeet Dokal, Julie Bungey, Peter Williamson, David Oscier, Jill Hows, and Lucio Luzzatto

Dyskeratosis congenita (DC) is a rare inherited disorder characterized by bone marrow failure, dystrophic changes in the skin and mucous membranes, and a predisposition to malignancy. The DC locus has been mapped to Xq28. The primary defect responsible for this disease remains unknown. We have studied four patients with this disease, three from one family and one from another. In all four patients, primary skin fibroblast cultures were abnormal both in morphology (polygonal cell shape, ballooning, and dendritic-like projections) and in growth rate (doubling time about twice normal). Fibroblast survival studies using four clastogens (bleomycin, diepoxybutane, mitomycin-c, and 4-nitroquinoline-1-oxide) and gamma radiation showed no significant difference between DC and normal fibroblasts. Cytogenetic studies performed on peripheral blood lymphocytes showed no difference between DC and normal lymphocytes with or without prior incubation with clastogens. However, bone marrow metaphases from one of three patients and fibroblasts from two of four patients (who were the eldest of the 4) showed numerous unbalanced chromosomal rearrangements (dicentrics, tricentrics, and translocations) in the absence of any clastogenic agents. Cell-specific differences and a higher rate of chromosomal rearrangements in the older patients appear to correlate with the clinical evolution of the disease. These findings suggest that the DC defect predisposes DC cells to developing chromosomal rearrangements.

DeBauche et al18; and Pai et al12 found that DC cells (fibroblasts and lymphocytes) were hypersensitive to bleomycin.

In light of the inconclusive reports in the literature regarding the spontaneous chromosomal instability and response of DC cells to clastogens, we have undertaken cytogenetic and survival studies on cells from DC patients with and without prior incubation with clastogens. The work was prompted initially by the need to design a BM transplant (BMT) protocol for the index case. We found that the DC defect predisposes DC cells to developing chromosomal rearrangements in the absence of exposure to clastogens, and that fibroblasts from all patients were abnormal.

MATeRIALS AND METHODS

Cell Lines and Culture Conditions

Primary skin fibroblast cultures were established from punch biopsy specimens from four DC patients (3 from family A and 1 from family B) and four non-DC members of family A. Fibroblasts were grown at 37°C in 5% CO2 in a mixture of fetal calf serum. Two immortalized fibroblast lines (GM691419 and Ch RuZ0) established from patients with Fanconi's anemia were obtained from Dr Duckworth-Rysiecki and Dr Chaganti, respectively.

Fibroblast Survival Studies

Fibroblast survival studies using clastogens (bleomycin, diepoxybutane, mitomycin-c, and 4-nitroquinoline-1-oxide) and gamma irradiation were performed on early passage fibroblasts. Skin fibroblast cultures were subcultured in 24-well plates containing growth medium. Twenty-four hours later, when they were in the logarithmic growth phase, they were exposed to the appropriate concentration of clastogen either as a pulse (bleomycin and diepoxybutane) or continuously (mitomycin-c and 4-nitroquinoline-1-oxide). The cells were then washed twice with phosphate-buffered saline and reincubated in fresh medium for a further 10 days. Fibroblasts were then harvested with trypsin and stained with trypan blue. Viable cells (unstained cells) were counted using a hemocytometer.

Cytogenetic Studies

Chromosomal analyses were performed on BM and fibroblasts without prior exposure to clastogens.
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Fig 1. Fibroblast morphology. (A) Fibroblasts from a normal control (original magnification ×400). (B) Fibroblasts from a patient with DC (original magnification ×400) showing abnormal morphology (polygonal shape, ballooning, and dendritic-like projections).

Cytogenetic studies on peripheral blood (PB) lymphocytes were performed with and without prior exposure to clastogens.21

RFLP Genetic Linkage Study

An RFLP genetic linkage study was undertaken in family A using the DXS52(St14-1) probe and TaqI digestion as described by Connor et al.8

Patients

Family A

Patient 1. Patient 1 (the index case in family A) first presented at the age of 29 years in August 1989 with symptoms of anemia. He was found to have oral leukoplakia, nail dystrophy, and skin pigmentation. Investigations showed he had severe pancytopenia (hemoglobin [Hb], 3.2 g/dL; white blood cell count [WBC], 1.2 × 10^9/L; platelets, 6 × 10^9/L). A biopsy of the BM showed that it was very hypocellular, with approximately 10% myeloblasts. In January 1990, he was referred to the Hammersmith Hospital for matched unrelated donor (MUD) BMT, at which time his HLA-identical younger brother (patient 2) was also diagnosed to have DC. Because the fibroblast survival studies had shown that his cells were not hypersensitive to clastogens, he was conditioned with the same dose (50 mg/kg × 4) of cyclophosphamide as that used for patients with acquired aplastic anemia rather than the low dose (5 mg/kg × 4) used in FA and total body irradiation. He did not develop severe mucosal toxicity, but failed to engraft. As a salvage
procedure, he received a second BMT from his younger brother (patient 2), who had a normal blood count but poor in vitro growth in long-term BM cultures. He showed signs of early PB engraftment, but died from respiratory failure 3 weeks after his second BMT (March 21, 1990). The cause of the respiratory failure remained unknown. No pathogen was isolated from the bronchial lavage and he had no evidence of graft-versus-host disease (GVHD) or mucosal toxicity. It is possible that the underlying DC may have predisposed this patient to developing pulmonary complications posttransplant.  

**Patient 2.** Patient 2 was diagnosed when he was 26 years old while he was being assessed as a potential BM donor for his elder brother (patient 1). Like his brother, he had nail dystrophy, skin pigmentation, and oral leukoplakia. He was asymptomatic with a normal PB count. However, his BM culture studies showed a significant reduction in hematopoietic progenitors and long-term BM culture studies suggested a functional stem cell defect with normal stromal function. These studies suggest that he may develop severe BM failure in the future similar to that in his elder brother (patient 1).  

**Patient 3.** Patient 3 is the nephew of patients 1 and 2. He was investigated at the age of 12 years because the RFLP genetic linkage study had shown that his mother (the younger sister of patient 1) was a carrier, as she had inherited the same allele (a 4.5-kb Taq I fragment identified with St14-1 probe) as that segregating with DC (see Fig 4). Further investigations showed that patient 3 had inherited this allele (4.5 kb) from his mother and had early changes of nail dystrophy and skin pigmentation. He was otherwise clinically asymptomatic and had a normal PB count. However, his BM culture studies showed a significant reduction in hematopoietic progenitors: granulocyte-macrophage colony-forming units (CFU-GM) were 16.4 per 10^9/L; BFU-E were 4.5 per 10^5 mononuclear cells plated (normal range, 30 to 330).  

**Family B**  

**Patient 4.** Patient 4 was referred to the Hammersmith Hospital at the age of 15 years in May 1990 for consideration for MUD BMT because of a deterioration in his blood count (WBC, 2 x 10^9/L; platelets, 30 x 10^9/L). His BM cultures showed a significant reduction in hematopoietic progenitors: CFU-GM were 5.0 per 10^9 mononuclear cells plated; BFU-E were 7.5 per 10^9 mononuclear cells plated. Similar to the three patients in family A, he had nail dystrophy, leukoplakia, and skin pigmentation. In addition, he also had an esophageal stricture, partial deafness, hypogonadism, and mild mental retardation. No suitable MUD donor was found and he died in July 1991.  

**RESULTS**  

**Fibroblast Morphology**  

In all four patients, primary skin fibroblasts were abnormal in morphology (polygonal cell shape, ballooning, and dendritic-like projections; Fig 1A and B). The different fibroblast cultures were of similar ages because the skin biopsies from which they were established were all taken simultaneously. The growth rate was also abnormal for all DC fibroblasts. The doubling time for normal fibroblasts was 2 to 3 days, whereas for DC fibroblasts it was 6 to 10 days. In one obligate carrier (the mother of patients 1 and 2), the fibroblasts looked normal and the doubling time was 2 days (ie, indistinguishable from normal).  

**Sensitivity to Clastogenic Agents**  

Fibroblast survival studies using bleomycin (0 to 120 μg/mL for 2 hours), diepoxybutane (0 to 10 μg/mL for 1 hour), mitomycin-c (0 to 25 nmol/L continuously administered), 4-nitroquinoline-1-oxide (0 to 25 nmol/L continuously administered), and gamma radiation (0 to 250 rad as a pulse) showed no significant difference between DC and normal fibroblasts (Fig 2). This is in sharp contrast to FA fibroblasts, which, as expected, were hypersensitive to diepoxybutane (Fig 2A) and mitomycin-c (Fig 2B).  

**Cytogenetic Analyses**  

**PB**  

PB lymphocytes showed no difference (gaps or breaks) between DC and normal lymphocytes with or without prior incubation with diepoxybutane (0.1 μg/mL for 48 hours), mitomycin-c (5 μg/mL for 72 hours), or 4-nitroquinoline-1-oxide (10^-5 mol/L for 48 hours). This is in sharp contrast to cells from patients with FA, which show increased chromosomal gaps and breaks upon exposure to clastogens. In one of four patients (the eldest of the 4), a triradial configuration (Fig 3A) was seen in 1 of 30 metaphases without prior exposure to clastogens.
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BM

BM from one of three patients (the eldest of the 4) showed (Fig 3B) numerous unbalanced chromosomal rearrangements without prior incubation with any clastogens.

Fibroblasts

Fibroblasts from two of four patients (the 2 eldest patients) showed a variety of unbalanced chromosomal rearrangements (dicentrics, tricentrics, and translocations) in the absence of any clastogens. Fibroblasts from the other two patients showed no chromosomal rearrangements. The type of abnormalities seen in the two patients (patients 1 and 2) are shown in Fig 3C and listed in Table 1. In addition, inversion 8 [inv(8)(p11.2;q22)] was seen in all the metaphases from patient 2. This abnormality was also observed in his mother and, therefore, we believe it is unrelated to DC.

RFLP Linkage Analysis

A linkage study was undertaken in family A because the two sisters of patients 1 and 2 wished to know whether they were carriers of the DC allele. Because Connor et al. have shown no recombination between the DC gene and the RFLPs identified by St14-1 in nine informative meiotic events (maximal Lod score of 3.33 at zero recombination

Fig 3. Cytogenetic abnormalities. (A) Metaphase spread made from PB of the eldest patient (patient 1) showing a triradial configuration. (B) Metaphase spread made from the BM of patient 1 showing a ring chromosome.
Fig 3. (Cont'd) (C) Partial karyotype showing chromosomal rearrangements identified in fibroblast culture from patient 1. Two types of dicentric chromosomes and one tricentric resulting from rearrangements between chromosomes 9 and 12 are shown.

fraction), we used this probe in family A. In our study, we found that the fragments identified by the St14-1 probe after Taq I digestion of genomic DNA were informative (Fig 4). The mother of patient 1 was found to have the 4.5-kb and 4.8-kb fragments. Both patients 1 and 2 had inherited the 4.5-kb fragment. This meant that the DC allele was segregating with the 4.5-kb fragment and the younger sister the 4.5-kb fragment from the mother. This meant (assuming zero recombination) that the elder sister was normal and the younger was a carrier. This prompted us to study the 12-year-old son (patient 3) of the younger sister. He was found to have inherited the 4.5-kb fragment and therefore (assuming zero recombination) the DC allele from his mother. This was confirmed on clinical examination, as he had early changes of nail dystrophy and skin pigmentation. The RFLP study was extended to include other members of family A and this allowed us to identify further carriers (Fig 4) in this family. Unfortunately, in generation IV, the two female carriers are both homozygous for the 4.5-kb fragment. This makes it impossible to say whether their male children (generation V) are normal or have DC.

### DISCUSSION

PB lymphocytes from all DC patients (and obligate carriers) did not exhibit increased chromosomal breakage with or without prior incubation with clastogens. In addition, fibroblasts from all four patients were not hypersensitive to bleomycin, diepoxybutane, mitomycin-c, 4-nitroquinoline-1-oxide, and gamma radiation. This confirms the findings of some previous studies. However, some studies have shown increased sensitivity of DC cells to ionising radiation or to 4-nitroquinoline-1-oxide and bleomycin. These discordant findings regarding the response of DC cells to clastogens and radiation may result from genetic heterogeneity in the disease. Therefore, in a given patient it is impossible to predict the sensitivity to clastogens and whether a patient is to receive high-dose chemoradiotherapy before a BMT. It is important to establish the sensitivity of that individual's cells to clastogens to ensure that the appropriate dose of chemotherapy is administered. In patient 1, the fibroblast survival studies and the lymphocyte cytogenetic studies had shown normal sensitivity to clastogens. He was therefore treated with the same dose of cyclophosphamide as that used for patients with acquired aplastic anemia rather than low-dose cyclophosphamide used in FA patients. This patient did not develop severe mucosal sensitivity posttransplant and, despite high-dose cyclophosphamide, rejected the first graft.

<table>
<thead>
<tr>
<th>Metaphases Examined</th>
<th>PB</th>
<th>BM</th>
<th>Fibroblasts</th>
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<td>Patient 1</td>
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<td>30</td>
<td>1</td>
<td>10</td>
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<tr>
<td>1 triradial</td>
<td>5</td>
<td>1</td>
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<td>29 46,XY</td>
<td>2</td>
<td>1</td>
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<tr>
<td>inversion 8'</td>
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<td>1 dicentric (9;12)</td>
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<td>1 dicentric (9;12)</td>
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<td>1 dicentric (12;22)</td>
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<td>1 Robertsonian translocation (14;15)</td>
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<td>1 tricentric isochoosome (12;9;12;+ isochoosome (9p)</td>
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<td>1 translocation (9;12), Robertsonian translocation (22;22)</td>
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<td>1 46,XY</td>
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<td>Patient 2</td>
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<td>30 46,XY inversion 8'</td>
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Inversion 8' (inv(8)(p11.2q22)) was seen in all metaphases from patient 2. This was also observed in the patient's mother, and we therefore infer that it is constitutional and unrelated to DC.

Abbreviation: R, ring chromosome.
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Fig 4. Pedigree of family A. The RFLP analysis for the individuals studied using the St14-1 probe and Taq I digestion are indicated. (○) Female carriers; (■) male affected members. The index case in this family is indicated by the arrow. He was found to have inherited the 4.5-kb fragment from his mother. The disease was therefore segregating with this 4.5-kb fragment. This RFLP analysis was informative up to generation V and allowed us to identify female carriers and one individual (patient 3) with DC before he had presented with clinical abnormalities. Because the mother of the boy (age 2 years at time of analysis) in generation V was homozygous for the 4.5-kb fragment, it was not possible to say whether this boy had DC.

suggesting the presence of residual, functional host lymphocytes after therapy. In this case, therefore, the in vitro studies correlated well with the in vivo observation.

Fibroblasts from all four patients were abnormal in both morphology and growth rate. Furthermore, fibroblasts from two of the four patients (the 2 older patients) showed numerous unbalanced chromosomal rearrangements in the absence of any clastogens. The elder of these two patients (patient 1) also had similar chromosomal abnormalities (but less numerous) in the blood and BM, with morphologic evidence for leukemic transformation. The demonstration of unbalanced chromosomal rearrangements in the PB, BM, and fibroblasts in the oldest patient and in the fibroblasts alone in the second oldest patient suggests a primary defect that predisposes DC cells to developing chromosomal rearrangements. These findings of cell-specific differences and of a high rate of chromosomal rearrangements in the two older patients appears to correlate with the evolution of the disease in vivo.

In our linkage study, we found that the fragments identified by the St14-1 probe after Taq I digestion of genomic DNA were informative and allowed us to correctly identify carriers and patient 3 in family A. Thus, this study confirms the findings of Connor et al6 and strengthens the linkage between the DC locus and the RFLPs identified by St14-1. Furthermore, this RFLP linkage analysis clearly shows that, in family A, DC is segregating as an X-linked recessive trait.

Although patients with FA and DC share some features in common (eg, BM aplasia), they appear to differ in two fundamental ways. Firstly, unlike Fanconi cells, in our four patients, DC cells were not hypersensitive to clastogens. Secondly, the primary defect in DC appears to predispose cells to developing chromosomal rearrangements rather than the chromosomal gaps and breaks seen in FA.

ACKNOWLEDGMENT

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REFERENCES

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