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Simultaneous measurement, using flow cytometry, of radiosensitivity and defective mitogen response in ataxia telangiectasia and related syndromes

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Abstract. In a retrospective study, peripheral blood mononuclear cells from 13 patients with known ataxia telangiectasia (AT) (Louis Bar syndrome, McKusick # 20890) were irradiated with different doses of X-rays prior to stimulation with phytohaemagglutinin. Mitogen response and cell cycle progression were assessed by two-parameter 5-bromo-2′-deoxyuridine/Hoechst – ethidium bromide flow cytometry. Compared to age-matched controls, AT cells show a severely defective mitogen response in both unirradiated and irradiated cells. Following irradiation with 1.5 Gy, AT cells exhibit significantly greater accumulations of cells in the G2 phase of the first cell cycle than controls. The ratio between the number of cells accumulated in the first cycle G2 phase and the growth fraction provides a clear distinction between AT and control cultures. In addition, two patients with microcephaly, normal intelligence, immunodeficiency, chromosomal instability and risk for lymphoreticular malignancies (Seemanová syndrome) and two patients with the Nijmegen breakage syndrome (both syndromes are listed as McKusick # 25126) also exhibit very poor mitogen response and moderately increased G2 phase accumulations after X-irradiation. The simultaneous assessment of radiosensitivity and mitogen response in a single cell kinetic assay provides a speedy and accurate classification of cells of AT and AT-related syndromes.

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Abbreviations: AT = ataxia telangiectasia; BrdU = 5-bromo-2′-deoxyuridine; GF = growth fraction; PHA = phytohaemagglutinin

Key words: Ataxia telangiectasia – Radiosensitivity – Immunodeficiency – Flow cytometry – Cell cycle

Introduction

Classical ataxia telangiectasia (AT) is an inherited disorder of ionizing radiation sensitivity with chromosomal instability and altered immune system functions. Clinical manifestations include progressive cerebellar ataxia with onset in late infancy, capillary dilatations, and elevated serum α-fetoprotein levels [12]. A number of other conditions in man show radiation sensitivity, most notably the Seemanová syndrome and the Nijmegen breakage syndrome. The latter patients lack the classical triad of ataxia, telangiectasia and elevated serum α-fetoprotein [18]. Although the gene locus for classical AT (complementation group C) has been mapped to chromosome 11q22–q23 [5, 26], the gene has not been identified to data. Due to extensive clinical heterogeneity of AT and AT-related syndromes, it can be anticipated that multiple mutations will be identified in the future. Conventional cell function tests which assess hallmarks of the AT cellular phenotype will therefore remain important.

In cultured skin fibroblasts from AT patients, increased radiosensitivity was established as reduced colony forming ability following exposure to gamma-rays [20] and X-rays [8]. This assay requires a skin biopsy sample and extensive cell culture. In peripheral blood lymphocytes, increased radiosensitivity has been determined by evaluation of chromatid type damage following irradiation of

cells in the G0- [7, 21] as well as in the G2 phase of the cell cycle [19]. The latter procedure requires extensive cytogenetic scoring of patient and reference cultures. Another diagnostic assay, using irradiated peripheral blood lymphocytes, involves individual cell cloning and thus requires 10–14 days for completion [6]. More recently, studies using flow cytometry have demonstrated marked accumulations of irradiated AT cells in the G2 phase of the cell cycle [2]. With one exception [15], all previous flow cytometric studies have employed fibroblast cultures or lymphoid cell lines from AT patients but not primary blood lymphocytes, that could be of immediate diagnostic use. Dose dependent accumulations of cells in the G2 phase of the cell cycle are the most immediate manifestations of the cellular radiation response [4, 10]. In the present paper we describe a novel flow cytometric approach [14] by which we measure radiation-induced G2 phase accumulations in peripheral blood mononuclear cells from AT patients.

The altered immune functions in AT involve both T- and B-cell lineages. The pattern of immunodeficiency suggests disturbed maturation of immunocompetent cells. In addition to frequently noted abnormalities of thymic development, a disturbance of the T-cell lineage has recently been confirmed by the demonstration of an increased ratio of gamma/delta over alpha/beta bearing T-cells [3]. A high proportion of cells with immature T-cell receptor types could be one of the reasons why peripheral blood mononuclear cells from AT patients exhibit a severely reduced response to standard mitogens such as phytohaemagglutinin (PHA) [11]. The unique aspect of the present study is that we examine both radiosensitivity and defective mitogen response, i.e. a very high proportion of cells which do not respond to mitogenic stimulation in a single flow cytometric assay. This is possible because each of these intrinsic features of the AT cellular phenotypes causes independent cell kinetic abnormalities which can be simultaneously assessed by flow cytometry. In addition to providing new insights into the cell kinetic consequences of radiosensitivity and defective mitogen response in AT, our results show that the simultaneous assessment of these cell kinetic parameters can be used as a speedy and reliable diagnostic test for AT and some of the AT-related syndromes.

Materials and methods

Blood donors

In order to assess the validity of our assay, only such patients were tested in whom the diagnosis of AT had been firmly established by the presence of at least five of the following criteria: typical neurological findings, ocular telangiectasia, elevated α-fetoprotein, altered immunoglobulin profiles, elevated chromosome breakage rates and/or clonal chromosomal changes involving chromosome 14q, and increased X-ray sensitivity [8, 13]. A group of 13 patients fulfilling these criteria were included, ranging in age from 1.5 to 31.5 years (mean 14.4 ± 10.1). A cohort of 33 healthy individuals (recruited from the authors and their families) served as age-matched controls (ages 1–35 years, mean 13.8 ± 10.1). Clinical data of two patients with the Seemanová syndrome and two patients with the Nijmegen breakage syndrome were previously published [1, 17, 23].

Cell culture

Peripheral blood mononuclear cells were isolated from 5–10 ml heparinized venous blood via the Ficoll gradient technique (density 1.077 g/ml; Ficoll-Paque, Pharmacia, Uppsala, Sweden). For cell culture, cell aliquots were adjusted 30 min after irradiation to concentrations of 1×10^5 cells/ml in RPMI 1640 medium (Gibco, Grand Island, NY, USA) and supplemented with 1% autologous serum, 15% heat-inactivated fetal calf serum (Gibco, or Roth, Karlsruhe, FRG), 1×10^{-4} *M* 2′-deoxycytidine (Sigma, Deisenhofen, FRG), 2×10^{-5} *M* α-monothioglycerol (Sigma), and 1×10^{-4} *M* 5-bromo-2′-deoxyuridine (BrdU) (Sigma). Cells were stimulated using 180 μg/ml PHA (Gibco) or 1.2 μg/ml PHA (Wellcome Diagnostics, Burgwedel, FRG). After 72 h at 37.5°C in 5% CO_2 incubators culture aliquots were harvested. The samples were centrifuged and resuspended in freezing medium containing RPMI 1640, 10% fetal calf serum and 10% dimethyl sulphoxide, and stored frozen at −20°C until analysis.

X-irradiation

X-irradiation was performed directly after Ficoll gradient isolation while cells were suspended in RPMI 1640 medium containing 1% autologous serum. Cultures were irradiated at ambient temperature using a Müller MG 150 generator (CHF Müller GmbH, Hamburg, FRG) at 110 kV and 10 mA (5 mm aluminum filter) yielding a dose-rate of 1.0 Gy/min. Parallel cultures were exposed to 0, 1.5 and 4.0 Gy.

Flow cytometry analysis

For flow cytometry, cells were thawed, centrifuged and resuspended at 4×10^5 cells/ml in a solution of 0.1 *M* Tris-HCl, pH 7.4, 0.154 *M* NaCl, 0.5 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1% NP40 and 0.2% bovine serum albumin (Sigma). For dual parameter analysis, cells were stained with 1.2 μg/ml Hoechst 33258 (Sigma), incubated for 15 min at 4°C, then stained with 1.5 μg/ml ethidium bromide (Serva, Heidelberg, FRG) and incubated for another 15 min at 4°C. Bivariate flow cytometric analysis was performed with an arc-lamp flow cytometer (ICP-22; Phywe AG, Göttingen, FRG) interfaced to a PDP 11/23 microcomputer (Digital Equipment Corp., Maynard, MA, USA). Quantitative cell cycle analysis was performed using personal computers and software programs developed by P.S. Rabinovitch (University of Washington, Seattle, WA, USA; "Multi2d" and "Mcycle", Phoenix Flow Systems, San Diego, CA, USA). Statistical analysis was performed using Student's *t*-test. All samples were coded with three letter codes and assayed blindly, the operator of the flow cytometer being unaware of the clinical diagnosis.

Results

Cell cycle distribution 72 h after exposure to X-rays

Figure 1 shows isometric plots of 72 h cell cycle distributions of mononuclear blood cells from a 12-year-old patient affected by AT *(left panels)* and from an age-matched healthy control donor *(right panels)*. After stimulation with PHA, activated cells enter the cell cycle and pass through three to four cell cycles by 72 h. Due to the quenching effect of BrdU (which is incorporated into DNA during semiconservative DNA replication), cells of the S and G2 phase of the first cell cycle (G2) exhibit a reduced fluorescence emission of the Hoechst 33258

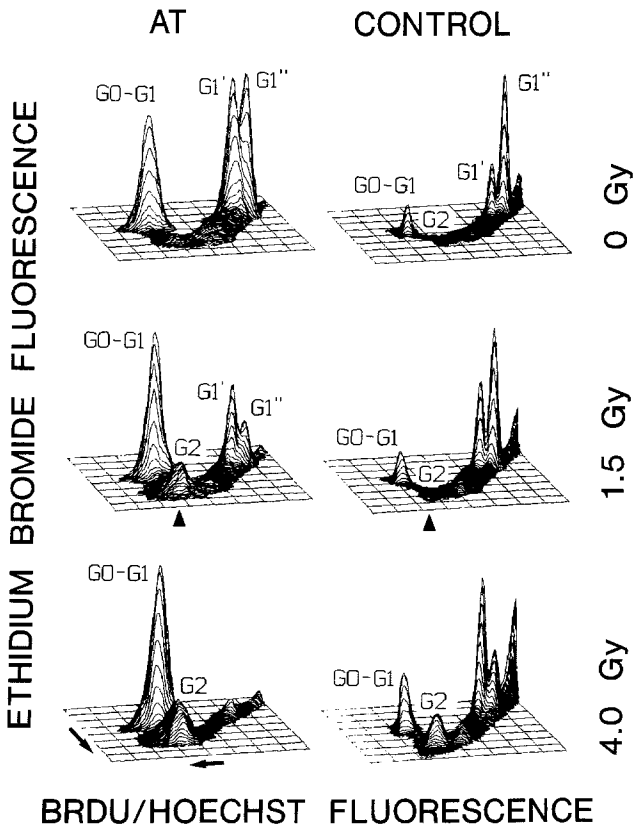

Fig. 1. Isometric plots of PHA-stimulated lymphocytes from a patient with AT *(left panels)* and an age-matched control *(right panels)*. The *upper row* represents unirradiated (0 Gy) samples, the *middle* and *lower rows* show the cell cycle distribution after exposure to 1.5 Gy and 4.0 Gy, respectively. Each cytogram represents the BrdU/Hoechst 33258 and the ethidium bromide fluorescence of a 72 h culture and is rotated by 194° with respect to the electronic signal origin (increasing fluorescence is given by *arrows*). The nomenclature of the three cell cycles displayed is as follows: G0–G1 and G2 denote the respective cell cycle phases of the first cell cycle; G1′ and G1″ stand for the second and third cell cycle G1 phases. The G2 phases of the first cell cycle are marked by *arrows* in the cytograms irradiated with 1.5 Gy

dye but an increased fluorescence of the ethidium bromide dye. The S phase cells can be seen as a lane emerging to the right of the non-cycling G0–G1 cell fraction (G0–G1). After cell division, cells in the G1 phase of the second cell cycle (G1′) exhibit only half of the BrdU/Hoechst and ethidium bromide fluorescence of the G2 phase nuclei; therefore, these cells can be clearly distinguished from the original G0–G1 cell population. Cells in the S and G2 phase of the second cell cycle are represented by signal clusters extending from the G1 peak of the second cell cycle (G1′) towards the G2 peak of the first cell cycle (G2). After the second division, cells in the G1 phase of the third cell cycle (G1″) appear right to the G1 fraction of the second cell cycle.

Effect of X-irradiation on cell cycle distribution

The figure shows a series of panels demonstrating the effects of two doses of X-irradiation (1.5 Gy and 4.0 Gy) on cell cycle progression of patient and control cells. The AT patient exhibits a high non-proliferating G0–G1 fraction *(upper row, left panel)*, whereas the control donor of the same age displays a much lower non-proliferating G0–G1 cell fraction *(upper row, right panel)*. It is important to note, however, that the non-irradiated, proliferating cells of both types of donors progress through three consecutive cell cycles without any noticeable arrest in the consecutive cycle compartments.

Increased radiosensitivity of the AT patients

Following irradiation with 1.5 Gy, a strong increase of the G2 phase cells of the first cycle occurs in the AT patient *(middle row, left panel)*. The increase of the G2 phase in the control donor is much less *(middle row, right panel)*. Exposure to 4.0 Gy causes a further increase in the G2 phase of the first cell cycle *(lower panels)*. At this radiation dose, only very few AT cells reach the second cell cycle, since the high proportion of non-cycling G0–G1 cells remains high and most cycling cells accumulate in the first cycle G2 phase compartment. Likewise, cells of the control donor respond to 4.0 Gy irradiation with a moderate G2 phase arrest and a moderate elevation of the non-cycling G0–G1 fraction; the inhibition is less severe, however, compared to the AT patient, and more control cells reach their second and third cell cycles *(lower row, right panel)*.

Assessment of mitogen response and radiosensitivity

Table 1 summarizes the quantitative data extracted from the type of analysis shown in Fig. 1 for each of the 13 AT patients and the age-matched controls. The non-proliferating G0–G1 fraction is significantly elevated in AT patients, regardless of whether AT cells were or were not exposed to X-rays. The second remarkable difference between AT patients and healthy controls concerns the degree of increment of G2 phase cells present in the first cell cycle after irradiation. At 1.5 Gy, the G2 phase fractions of AT patients increase three-to-fourfold, whereas the increment is considerably less in controls (Table 1). At higher doses of irradiation (4.0 Gy), the G2 phase of the control group continues to increase, whereas AT cells show only a moderate further increment of the G2 phase fraction. The ratio between the G2 phase fraction

Table 1. Quantitative cell cycle analysis

		G0–G1	G2	G2/GF
AT	0.0 Gy	62.1 ± 15.4	4.3 ± 1.9	0.12 ± 0.03
	1.5 Gy	68.4 ± 14.5	14.4 ± 8.2	0.44 ± 0.11
	4.0 Gy	73.8 ± 15.4	16.0 ± 12.9	0.52 ± 0.19
Control	0.0 Gy	25.2 ± 11.8	2.9 ± 1.0	0.04 ± 0.02
	1.5 Gy	27.2 ± 11.7	5.9 ± 2.0	0.08 ± 0.03
	4.0 Gy	37.3 ± 15.4	17.2 ± 6.8	0.28 ± 0.09

Percentages of cells in G0–G1 and G2 phase of the first cell cycle, and G2/GF ratio of 13 AT patients and 33 age-matched donors *(control)* at 72 h after irradiation with different doses of X-rays. The values are mean ± SD

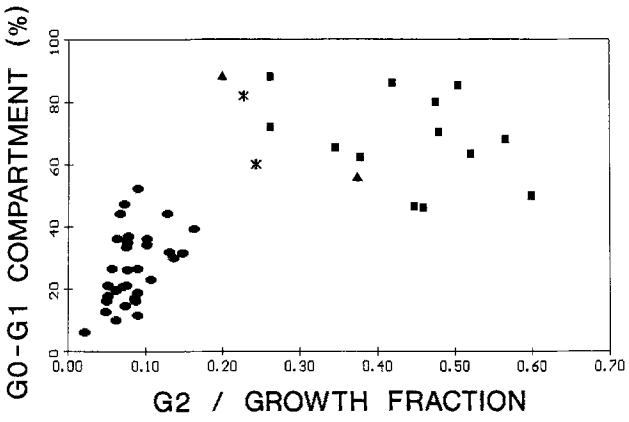

Fig. 2. Diagram of G2/GF ratio versus the non-proliferating G0-G1 fraction of 72 h after X-irradiation with 1.5 Gy. *Solid squares* denote AT patients; *solid circles* denote age-matched healthy control donors. The *stars* represent two patients with the AT-related Seemanová syndrome. The *triangles* denote two patients with the Nijmegen breakage syndrome

of the first cell cycle and the overall growth fraction (GF) (which includes all proliferating cells except the G0–G1 fraction) is significantly elevated in AT patients following exposure to 1.5 Gy compared to the control group ($P < 0.001$).

Classification of AT patients by increased G2/GF ratio and high non-proliferating fraction

Figure 2 depicts the G2/GF ratio plotted against the non-cycling G0–G1 fraction following exposure to 1.5 Gy. The control donors show significantly fewer cells in the non-cycling (G0–G1) compartment than AT patients at 72 h after stimulation ($P < 0.001$). There is only a small region of overlap between controls and AT patients with respect to their G0–G1 cell fractions. The second parameter, the G2/GF ratio (which measures radiosensitivity), separates all AT patients from the controls. The combined measurement (G2/GF ratio vs. the non-proliferating fraction) thus permits the unambiguous classification of AT cells. Moreover, cells from two patients with the Seemanová syndrome, and cells from two patients with the Nijmegen breakage syndrome exhibit high non-proliferating G0–G1 fractions, comparable to those seen in classical AT. Cells from these AT-related syndromes show somewhat lower radiosensitivity than cells from classical AT patients. Nevertheless, their G2/GF ratio is increased and outside the 99% confidence limits for the group of controls.

Discussion

The present study confirms that the primary cell kinetic manifestation of increased radiosensitivity in classical AT results in massive G2 phase accumulations. Although our data show that exposure of peripheral blood mononuclear cells to X-rays also causes G2 phase accumulations in age-matched controls, the effects seen in AT patients are far more severe, permitting a clear distinction between healthy and affected individuals. The molecular reason for the increased radiosensitivitiy in AT is not known, although recent assays using damaged recombinant DNA molecules suggest that the effectiveness of the repair of double strand breaks may be reduced in AT [22]. Our data clearly refute earlier claims that the radiosensitivity of AT cells may be due to their failure to become sufficiently delayed in the G2 phase compartment of the cell cycle in order to be repaired [13, 25]. Also, our data rule out any type of defect involving a specific cell cycle repair gene whose function would be to arrest cells in the G2 phase of the cell cycle. If such a gene (like the yeast RAD9 gene) [24] exists in human cells, it appears to function properly in AT.

The response of human peripheral blood mononuclear cells to polyclonal stimulation by lectins is known to vary greatly as a function of a number of intrinsic and extrinsic factors. One such factor is donor age: 72 h blood cultures from children show a much more synchronous response to polyclonal activation and mobilize a far greater proportion of their resting mononuclear cells than seen in adults [9, 16]. In our cohort of control individuals ranging in age from 1 to 35 years the size of the non-cycling (G0–G1) cell fraction (irradiated with 1.5 Gy and measured at 72 h after stimulation) ranged from 6% to 53%. In contrast, our AT patients of comparable age distribution typically had G0–G1 phase fractions in excess of 50% (range 43%–88%); a consistently elevated fraction of non-cycling cells at 72 h after stimulation thus marks the AT group irrespective of patient age. As mentioned before, this uniformly poor response of AT mononuclear blood cells to mitogenic stimulation is likely to be one of the manifestations of the intrinsic immune defect of these patients. A poor mitogen response was also observed in the patients with Seemanová syndrome and Nijmegen breakage syndrome, which is in agreement with their immunodeficiency [1, 17, 23].

In this retrospective analysis, using blood specimens from previously well characterized patients, the simultaneous assessment of radiosensitivity and mitogen response in a single flow cytometric assay led to a clear distinction between patient and control cells. As a next step, we plan to apply our assay in a prospective study. Given its speed and relative simplicity in laboratories with standard flow cytometric instrumentation, we believe that our assay will permit a far more liberal screening of patients belonging to the wide spectrum of AT-related phenotypes. In view of the heterogeneity of AT-related manifestations in children and young adults we would be able to define additional categories of syndromes which, for instance, might be discordant rather than concordant with respect to radiosensitivity and mitogen response.

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