Repair of Ionizing Radiation DNA Base Damage in Ataxia-Telangiectasia Cells

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ABSTRACT

Micrococcus luteus endonuclease sensitive sites were measured by alkaline elution in normal human and ataxia-telangiectasia (AT) fibroblasts after ionizing radiation. Due to the sensitivity of this assay, repair of base damage after 3 to 6 kilorads has been measured after oxic or hypoxic radiation. With 5.5 kilorads of oxic radiation, more than 50% of the base damage was removed after 1.5 h of repair incubation in all cells, including exr+ and exr- AT cells, and approximately 75% was removed by 4 h. After 3 or 4.5 kilorads of hypoxic X-irradiation, repair was equivalent in normal and exr- AT cells. This study included three exr- AT strains which have been reported to be deficient in the removal of γ-ray base damage at higher doses. Since these strains repaired ionizing radiation base damage normally at lower doses, which are more relevant to survival, it is concluded that the X-ray hypersensitivity of AT cells is probably not related to the repair of base damage.

INTRODUCTION

AT2 is an inheritable autosomal recessive disease characterized by certain neurological disorders; ocular and cutaneous telangiectasia, particularly in sun exposed areas; various immunodeficiencies; and a predisposition to cancer, especially for the lymphoproliferative type (1). In cells from AT patients, there is an increased frequency of spontaneous chromosome breakage and other chromosome aberrations (reviewed in Ref. 1). The frequency of X-ray induced chromosome aberrations is also increased in AT cells compared to normal. Some AT patients who develop lymphoma exhibit a striking clinical hypersensitivity to radiotherapy of irradiated skin and other normal tissues (1). Cultured fibroblasts and lymphocytes from AT patients show an increased sensitivity to ionizing radiation and certain chemical agents (1, 2). The basis for this radiation hypersensitivity in AT cells is not clearly understood. Proposed mechanisms for the defect(s) in AT cells can be divided into two general areas: an abnormality in chromatin organization affecting DNA synthesis and repair; or a specific abnormality in DNA repair.

A consistent abnormality observed in AT cells has been the failure of these cells to demonstrate an inhibition of DNA synthesis after X-irradiation as found in normal cells (3). A further indication for an abnormality in DNA synthesis is the prolonged S phase and decreased growth rate in AT cells compared to other human cells (3). The genetic heterogeneity of AT is demonstrated by cell fusion studies where AT cell strains have been assigned to several complementation groups based on reversal of the above abnormalities of DNA synthesis (4, 5). The failure of AT cells to demonstrate inhibition of DNA synthesis following irradiation can be mimicked in normal cells by treatment with agents which alter chromatin structure (6). A difference in chromatin structure between normal and AT cells is also supported by the observation that exposure to DNase II in permeabilized cells produces more strand breaks in AT cells (7).

Since AT cells are hypersensitive to X-rays and several X-ray-mimetic chemical agents (1, 2, 8–13), the possibility for a specific DNA repair defect, analogous to xeroderma pigmentosum with UV and UV-mimetic agents, has been investigated in many laboratories. X-rays and most other agents to which AT cells are hypersensitive produce DNA strand breaks, probably via a free radical mechanism (2, 10, 12, 14), but repair of these strand breaks is normal in AT cells (1, 8, 15). Paterson et al. (8) have found that some AT cell strains (exr-) exhibit decreased repair replication after high dose hypoxic γ-irradiation suggesting a defect in excision repair. The exr+ cells also show decreased repair replication after high doses of N-methyl-N’-nitro-N-nitrosoguanidine (13). AT strains with normal repair replication (exr+) fall in different complementation groups compared to exr- cells as measured by DNA synthesis rates (4, 5). Extracts from AT cells have been found to have lower priming activity for DNA polymerase on X-irradiated DNA templates compared to other cells (16, 17). However, AT cell extracts were found to have normal levels of endonuclease activity for γ-irradiated DNA (18) and to have normal capacities to excise γ-ray induced thymine base damage (14). In intact cells, γ-ray base damage was found to be more persistent in exr- AT cells than normal and exr+ AT cells using a crude endonuclease preparation from Micrococcus luteus (8). This assay involves digesting DNA purified from normal or AT cells with the endonuclease preparation and then determining the frequency of strand scissions produced by the endonuclease(s) by alkaline sedimentation. Since the frequency of γ-ray base damage is the same order of magnitude as the frequency of DNA single strand breaks produced by γ-rays, cells had to be irradiated with a high dose of γ-rays, 50 kilorads, under hypoxic conditions to reduce the frequency of single strand breaks. Recently, an approach has been developed to measure ESS by alkaline elution (19). With this approach, ESS can be measured to be less than 0.5/10⁹ daltons of single strand DNA. We have studied the repair of ionizing radiation base damage with this technique in normal and AT cells at substantially lower doses than the original study (8) to determine the extent of the defect in exr- AT cells with more biologically relevant doses of radiation.

MATERIALS AND METHODS

Cells and Cell Labeling. Attempts were made to use the original AT fibroblast cell strains assigned to exr+ and exr- groups by Paterson et al. (1, 8). These strains have a limited life span in culture and most commercial sources have been depleted. The exr- strains used were AT2BE and AT3BI which were provided by R. Reynolds and originated from M. Paterson and AT97CTO which was provided by C. Arlett. The exr+ strain, AT5BI, was provided by H. Nagasawa. The AT strain, AT19JE-F (GM2052), and the normal human fibroblast strain, AG1522, were obtained from the Institute for Medical Research, Camden, NJ. Fibroblasts were grown in F-12 medium (GIBCO) supplemented with 20% fetal calf serum, penicillin, and streptomycin. Cells (2 × 10⁵) were seeded on 60-mm dishes and grown for 3 days with [14C]thymidine (0.05 μCi/ml) and then replaced with nonradioactive medium for an additional 3 days at which time they were in a confluent confluence.
monolayer. Cells were used at passages 14–15 for AT2BE, 14–16 for AT3BI, 14–17 for AT97CTO, 12–14 AT19UJE-F, and 11–31 for AG1522. All the AT strains grew markedly more slowly than the normal strain. The incorporation of labeled thymidine during the 3-day labeling period provided a rough estimate of the growth rates. The incorporation of labeled thymidine in the AT cells was consistently delivered with a $^{137}$Cs source at 1.2 kilorads/m at 37°C. For hypoxic irradiation, cells were gassed with 95% N$_2$-5% CO$_2$ for 1 h at 25°C in glass plates with agitation; the effluent O$_2$ concentration was less than 10 ppm (9). Hypoxic samples were irradiated with a 15-MeV photon beam of a linear accelerator at 1 kilorad/min. L1210 cells were $\gamma$-irradiated in medium with 1 kilorad at 0°C as described previously (19).

Alkaline Elution. The procedure used is similar to that described previously (19) with some modifications. Briefly, 2 x 10$^5$ fibroblasts were filtered onto a 25-mm-diameter 0.8-$\mu$m-pore size polycarbonate filter (Nuclepore) with cold buffered saline consisting of (g/liter): CaCl$_2$, 0.1; KCl, 0.2; KH$_2$PO$_4$, 0.2; MgCl$_2$•6H$_2$O, 0.1; NaCl; 8; Na$_2$HPO$_4$•H$_2$O, 2.16. Aerobic irradiation was performed at a $^{137}$Cs source at 1.2 kilorads/m at 37°C. For hypoxic irradiation, cells were gassed with 95% N$_2$-5% CO$_2$ for 1 h at 25°C in glass plates with agitation; the effluent O$_2$ concentration was less than 10 ppm (9). Hypoxic samples were irradiated with a 15-MeV photon beam from a linear accelerator at 1 kilorad/min. L1210 cells were $\gamma$-irradiated in medium with 1 kilorad at 0°C as described previously (19).

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Radiation base damage detected as ESS was quantitated by

$$
\text{Net } e = E(\gamma, \text{no endo}) - E(\text{control}, \text{endo}) + E(\gamma, \text{no endo}) - E(\text{control}, \text{endo})
$$

where $e$ is the log of the fraction of DNA retained after 12 min of elution, $\gamma$ is the irradiated sample, and endo is samples digested with endonuclease (19).

The strand break frequency has been shown to be proportional to a first order function of the elution rate (22); the value $E$ is a measure of the slope or rate of elution on a first order plot (19, 22). By subtracting out the effect produced by the endonuclease alone and single strand breaks present without endonuclease digestion, an estimate, $e$, of the ESS frequency can be obtained (19). This value, $e$, is proportional to the dose of radiation used when the cells are analyzed immediately after irradiation (19). The actual frequency of ESS was estimated by

$$
(2.7/10^9 \text{ daltons}) \times (\text{net } e)
$$

where the last value represents the elution of LI210 cells which had been $\gamma$-irradiated and (2.7/10$^9$ daltons) is the single strand break frequency produced by 1 kilorad in LI210 cells (22).

**RESULTS**

The alkaline elution technique has been shown to be a sensitive assay for the measurement of DNA single strand breaks in mammalian and other higher eukaryotic cells (22). In the standard assay, up to approximately 500 rad equivalents of single strand breaks (1.4 breaks/10$^9$ daltons) can be measured (22). By increasing the pump speed, this range can be increased to 10$^4$ rad equivalents or about 25 single strand breaks per 10$^9$ daltons (19, 23). If the cellular DNA on the filter is digested with a protein extract from $M$. *luteus* prior to alkaline elution, base damage in the DNA is cleaved by damage specific glycosylases and endonucleases in the *M*. *luteus* preparation (19). Very low levels of base damage can then be measured by alkaline elution. At high pump speeds, 0.7–0.8 ml/min, base damage produced by up to 10 kilorads of X-rays or up to 0.8 J/m$^2$ of far UV can be quantitated (19). At this pump speed, base damage can be measured to fewer than 0.5 ESS/10$^9$ daltons of single strand DNA (19); the major limitation on sensitivity is the level of nonspecific endonuclease activity in a particular preparation.

In Fig. 1, typical results for such experiments are shown after oxic irradiation with 5.5 kilorads of $\gamma$-rays. The cells were incubated for 4 h after irradiation such that most of the direct
(nonenzymatic) single strand breaks had been repaired in both cell types. For comparative purposes, the effect of 1 kilorad on L1210 cells at 0°C without repair is included. Under these conditions, 2.7 direct single strand breaks per 10⁹ daltons have been shown to be produced (22). When the endonuclease digestion is included, a marked increase in elution occurs in the γ-irradiated samples. This indicates that a substantial frequency of damaged bases remains in both normal and AT cells at this repair time. The level of γ-ray ESS was similar in both cell types. With the endonuclease preparation, a significant level of nonspecific ESS is detected. The level of nonspecific ESS varied in different experiments and cell strains, but typically 75–85% of the DNA was retained on the filter after 12 min of elution.

In Fig. 2, data are pooled from all the experiments done with AT cells. The level of ESS in irradiated samples was determined by subtracting the effect produced by the endonuclease digestion alone and remaining radiation induced direct single strand breaks. Efforts were made to control for variability in culture conditions by using confluent cultures and by irradiation in buffered saline. When fibroblasts were irradiated suspended in buffered saline with 15 mM EDTA at 0°C, 1.0 kilorad of γ-rays produced 3.4 ESS and 4.0 direct single strand breaks per 10⁹ daltons of single strand DNA. The efficiency of ESS production is 3.4 × 10⁻¹² rad⁻¹ dalton⁻¹ or 3.4 × 10⁻¹⁶ Gy⁻¹ dalton⁻¹. The efficiency for ESS production at 50 kilorads by Patterson et al. (8) was 2.4 × 10⁻¹² rad⁻¹ dalton⁻¹. Initial ESS and direct single strand breaks were similar in normal and AT cells. As seen in Fig. 2, much of the γ-ray base damage is efficiently removed from cells in the first several h of repair incubation. By 4 h of repair, the level of ESS produced by 5.5 kilorads is similar to that initially produced by 1 kilorad. In both AT cells and the normal strain, more than 50% of the base damage is removed within 1.5 h and approximately 75% by 4 h. At later repair times, the level of ESS remaining was very low in both normal and AT cells. For completeness, three points are included at the 4-h repair time where no ESS were recognized; this can probably be attributed to SDS remaining on the filter since the elution of the internal standard (3H cells) was also reduced.

This problem was attributed to blockage (bubbles) in the outflow tract since modification of the apparatus reduced the incidence. When this problem was more frequent in earlier experiments with normal cells (data not shown), no ESS was detected in samples affected by this problem. On the right side of Fig. 2, the frequency of ESS is shown after hypoxic X-irradiation and repair incubation. No difference was seen between AT and normal cells.

Even though confluent cells were used in these experiments, the passage number or "replicative potential" of the cells had an apparent modest effect on the initial rate of repair of γ-ray base damage. As seen in Fig. 2, earlier passage normal cells had a lower level of ESS 1.5 h after 5.5 kilorads. Five of the six lowest determinations were with passage 11 and 12 cells. When passage 31 normal cells were used, the level of ESS at this repair time was comparable to AT cells, even though these normal cells grew faster than AT cells as described in "Materials and Methods." The question arises which normal cells are the most appropriate control since the AT cells could not be maintained in culture past passage 20 while the normal cells were grown past passage 34. Repair of γ-ray ESS was similar in the exr⁺ strain and AT191JE-F and the three exr⁻ strains at this repair time. By 4 h of repair incubation, this difference seen with earlier passage normal cells was much less pronounced.

DISCUSSION

In this study, the level of ionizing radiation base damage recognized by an endonuclease extract from M. luteus was measured by alkaline elution in normal human and various AT fibroblast cell strains. At several repair incubation times, the frequency of ESS was similar in three exr⁺ AT cell strains, an exr⁻ AT strain, the AT strain AT191JE-F, and a normal skin fibroblast cell strain. Repair of ESS was studied after bothoxic and hypoxic radiation since AT cells have been shown to be hypersensitive to both; the oxygen enhancement ratio for cell killing is similar in normal and AT cells (9, 24). As seen by Paterson et al. (8) with 50 kilorads under hypoxic conditions, the frequency of ESS after 5.5 kilorads of oxic radiation decreases rapidly with repair incubation in normal and exr⁺ AT cells. However, at this lower dose (5.5 kilorads), we also observed rapid removal in the exr⁻ AT cells. Under hypoxic conditions, no difference was seen between normal and exr⁻ AT cells. When data were pooled from all experiments in Fig. 2, early passage normal cells tended to have less ESS 1.5 h after irradiation than the other cells and later passage normal cells, although more than 50% repair occurred in all cells by this time. Attempts were made to keep culture conditions constant by using confluent cells since marked differences in DNA repair have been reported during different phases of the cell cycle and replicative rate (e.g., Refs. 25 and 26). The effect of donor age or passage number on DNA repair capacity has been controversial, but many have found some decrease in DNA repair capacity (to varying extents) with increased age in culture and decreased rate of DNA replication (27). At the 1.5-h repair time, the level of ESS was equivalent in the AT cell strains and the later passage normal cells which as described earlier were a better match to the AT cells in both growth rate and number of passages before senescence. No difference was found between exr⁻ strains and the exr⁺ AT strain in either growth rates or repair of γ-ray ESS. By 4 h of repair, the level of ESS in early passage normal cells and the other samples was essentially equivalent.

Base damage produced by ionizing radiation consists of many...
different lesions and is produced at much lower levels than equitoxic doses of other agents which produce DNA base damage. For example, based on our efficiency for ESS produced by γ-rays, a $D_{10}$ (cell survival, 10%) dose of γ-rays would result in approximately 1.4 ESS/10⁹ daltons of DNA in normal cells and less than 0.4 ESS/10⁹ daltons in AT cells (2). With far UV, approximately 300 pyrimidine dimers/10⁹ daltons are produced by a $D_{10}$ dose in normal human cells (19). Even in xeroderma pigmentosum cells, which do not excise pyrimidine dimers, the $D_{10}$ dose is greater than 10 dimers/10⁹ daltons (19). The case is similar with monofunctional alkylating agents where greater than 10 DNA alkylations per 10⁹ daltons are produced with a $D_{10}$ dose in normal cells (19). Many different types of base damage occur with ionizing radiation (discussed in Ref. 28). If one assumes that only a subset of the total base damage is not repaired in AT cells, then the frequency of these particular lesions would probably be lower at equitoxic doses than the frequency of double strand breaks produced by X-rays or interstrand cross-links produced by bifunctional agents. It appears unlikely that a single strand lesion could produce a greater effect on cell lethality than double strand lesions.

From our results and others, it is unlikely that deficient repair of γ-ray base damage is the cause for the γ-ray hypersensitivity in AT cells. All AT cells have a very similar sensitivity to ionizing radiation (1, 2), even though they can be divided into exr− and exr+ groups based on repair replication or ESS removal after high doses of γ-rays (1, 2). Since the removal of ESS is similar in exr−, exr+, and normal cells at lower doses of radiation, one would have to speculate that a very infrequent type of base damage was responsible for the increased lethality in AT. AT cells are hypersensitive to a diverse group of DNA damaging agents including ionizing radiation (2), bleomycin (10), actinomycin D (29), N-methyl-N′-nitro-N-nitrosoguanidine (13), necarzinostatin (11), streptonigrin (12), 4-nitroquinoline 1-oxide (1), Adriamycin (12), H₂O₂ (12), and the bis-benzimidazole dye Hoechst 33342 (7); an unusual glycosylase would be needed to recognize all these agents. The most consistent abnormality found in AT appears to involve an abnormality in the regulation of DNA synthesis (3). It is not unreasonable to expect that the same abnormality may affect repair replication (8) after very high doses of ionizing radiation. The same explanation may apply for the defect (8) seen in the excision of base damage, ESS, in AT cells after similar high doses of γ-irradiation although this observation has not been confirmed in similar experiments (30).³

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