[CANCER RESEARCH 59, 298–300, January 15, 1999]

Advances in Brief

Asbestos and DNA Double Strand Breaks[1]

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Abstract

A radiosensitive DNA repair-deficient xrs-5 cell line was used to study asbestos cytotoxicity and DNA double strand breaks (DSBs). Although xrs-5 cells did not show any increase in sensitivity to chrysotile fibers in short-term (4-h) treatment when compared with wild-type CHO cells, longer-term exposure (24 h) gave significantly lower cell survival accompanied by a cell growth delay as well as a higher DNA DSB induction in this mutant cell line. These results suggest an important role played by DNA DSBs at the initial stage of asbestos injury.

Introduction

Although asbestos exposure has been implicated in the development of pulmonary fibrosis, bronchogenic carcinoma, and malignant mesothelioma in human and animal studies (1–4), the pathogenic mechanisms of asbestos are not fully understood. Asbestos fibers are cytotoxic as well as genotoxic in various cell types and known to induce DNA and chromosome damage (5). They induce DNA damage either by direct physical interaction or by indirect action of reactive oxygen species (6, 7). Previous studies have shown that asbestos fibers induce various forms of DNA damage in mammalian cells. DNA strand breakage produced by crocidolite fibers was demonstrated, for example, in C3H10T1/2 and rat embryo cells (8, 9). DNA damage caused by chrysotile as well as crocidolite was indirectly evaluated by unscheduled DNA synthesis in rat pleural mesothelial cells (10). Asbestos induced the oxidative DNA damage product 8-hydroxydeoxyguanosine in HL60 cells (11). Although the kinds of DNA damage mentioned above may have biological significance, there are few studies on DNA DSBs,[3] which are known to be the most significant form of DNA damage leading to cell lethality and transformation if left unrepaired (12). Marczynski *et al.* (13) reported that there are higher incidences of DNA DSBs in WBCs of occupationally exposed asbestos workers as compared with the nonexposed control population. However, there has been no appropriate laboratory experimental system measuring the production of DNA DSBs associated with asbestos exposure. One reason for the lack of DNA DSB measurement could be that the yield of this kind of damage is much lower than the other DNA damage, such as single strand breaks and base damage. In this study, we used a DNA DSB repair-deficient mutant cell line of CHO cells (xrs-5 cell line) to overcome the sensitivity problem associated with DNA DSBs. The xrs-5 cells were first isolated in the early 1980s and have a severe defect in DNA DSB repair and, as a result, these cells are extremely sensitive to ionizing radiation (14). Recent studies identified a protein (Ku 80) responsible for the xrs phenotype (15, 16). Using this cell line, we demonstrate that chrysotile asbestos induce DNA DSB in cultured cells exposed to asbestos for a 24-h treatment period.

Materials and Methods

Cell Culture and Colony Formation Assay. CHO and xrs-5 cell lines were kindly contributed by Dr. Hatsumi Nagasawa (Harvard University, Boston, MA; xrs-5 cells were originally isolated by Dr. P. Jeggo's laboratory, University of Sussex, Brighton, United Kingdom). Cells were maintained in F-12 medium supplemented with 10% fetal bovine serum and antibiotics in an humidified incubator at 37°C (5% CO_2). All experiments were performed with exponentially growing cells. For DNA DSB experiments, cells were labeled with 370 Bq/ml [^{14}C]dThd and 5 μM cold dThd for 48 h. For cell survival assay, an appropriate number of cells were plated after asbestos treatment such that 50–200 colonies would yield after 7–10 days. Colonies were stained with 10% giemsa for counting.

Growth Rate Determination. Cells were seeded at a density of 200 cells/well in a 96-well microplate (Falcon) with 100 μl of F-12 medium. One day later, 50 μl of supernatant was discarded and 50 μl of fresh medium was added in the control wells, and 50 μl of suspended fiber solution (which contained chrysotile fibers at 0.76, 1.52, and 3.04 μg/well) were added into the other wells. After a 24-h incubation at 37°C, supernatant in every well was removed, and culture was washed with fresh medium. Adherent cells were fixed with 10% formaldehyde for 30 min, stained with Giemsa/methanol (50% v/v), and counted under a light microscope.

Asbestos and γ-Irradiation. International Union Against Cancer standard reference Rhodesian chrysotile was received as a kind gift of Dr. Robert Nolan (Brooklyn College, New York, NY). A stock solution of 2.5 mg/ml was made in sterilized deionized water and used for all experiments. A ^{137}Cs γ-irradiator was used for irradiation at a dose rate of 6.7 Gy/min.

DNA DSB Assay. DNA DSBs were determined using CHEF gel electrophoresis assay, as described (17). After treatment with chrysotile and/or radiation, cells were trypsinized, washed, and the resultant cell pellets were immediately embedded in 0.5% agarose (Incert agarose; FMC Bioproducts) prepared in serum-free medium. These agarose samples were cut and immersed in an ice-cold lysis solution containing 0.5 M EDTA, 0.01 M Tris, 2% Sarcosyl, and 0.2 mg/ml proteinase K (pH 8.0) for 1 h, followed by an overnight incubation at 50°C. After overnight lysis, samples were washed for 1 h in 0.1 M EDTA, 0.01 M Tris (pH 8.0), and treated with 0.1 mg/ml RNase A for 1 h at 37°C.

Electrophoresis was carried out using 0.8% agarose gel in 0.5 × TBE buffer at 14°C in a CHEF gel box (Bio-Rad). The applied voltage was 200V with 60-s pulse time for the first 9 h, followed by a 120-s pulse time for the last 15 h. After electrophoresis, gels were stained and cut to separate the plug from the lane for each sample. The ^{14}C activity of each piece was measured in a scintillation counter, and the FAR was calculated as the disintegration/minutes of a lane divided by the total disintegration/minutes (lane + plug) per sample. The FAR value for nonirradiated control cells was 1–3%.

Statistical Analysis. All of the results in this study were obtained from two to three independent experiments, and the data were expressed as the mean + SE, unless indicated otherwise. A two-sample Student's *t* test was used to evaluate some of the critical data. The difference between means were regarded as significant if $P < 0.05$.

Results and Discussion

Conventional colony formation assay was used to examine the cytotoxicity of wild type CHO cells and the DNA DSB repair-deficient mutant xrs-5 cells, exposed to graded doses of chrysotile for

Received 10/5/98; accepted 11/24/98.

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[1] Supported by NIH Grants CA 71438, CA 43322, ES 05786, and ES 07890.

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[3] The abbreviations used are: DSB, double strand break; CHEF, clamped homogeneous electric field; FAR, fraction of activity released.

either 4 h or 24 h (Fig. 1). xrs-5 cells were slightly more sensitive to chrysotile as compared with CHO cells in a short-term (4 h) exposure, however, the difference may not be significant in this case. On the other hand, xrs-5 cells showed a clear sensitivity against wild type CHO cells in a longer term (24 h) exposure to chrysotile. As far as we know, this is the first study in which an ionizing radiation sensitive cell line is also sensitive to asbestos fibers. These data may also indicate that prolonged exposure produces more damage to cells than short-term exposure in asbestos fiber treatment (3, 18).

Because a significant difference in cytotoxicity was observed in the 24-h asbestos exposure between CHO and xrs-5 cells, we also examined the growth status of CHO and xrs-5 cells exposed to chrysotile for 24 h at 37°C. These results were obtained by counting the change in the number of cells after 24-h exposure to various concentrations of chrysotile using 96-well microplate. Fig. 2 shows the results of these

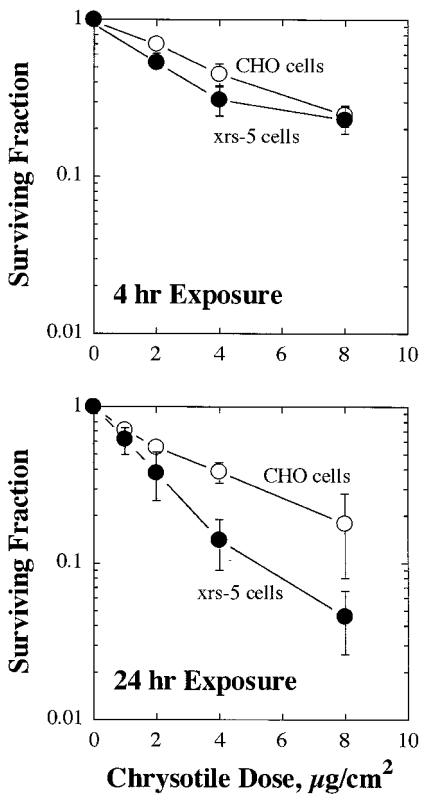

Fig. 1. Cell survival studies by colony formation assay in exponentially growing CHO and xrs-5 cells exposed to various concentrations of chrysotile fibers for 4 h and 24 h at 37°C.

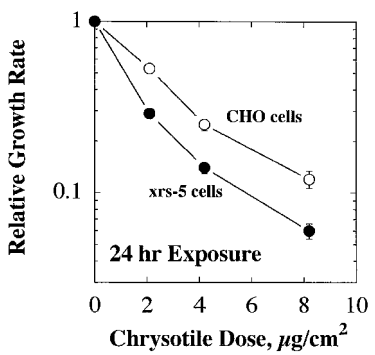

Fig. 2. Relative growth rate in CHO and xrs-5 cells exposed to various doses of chrysotile for 24 h at 37°C. Data are normalized for nontreated control (1.0). The results were obtained from three independent experiments.

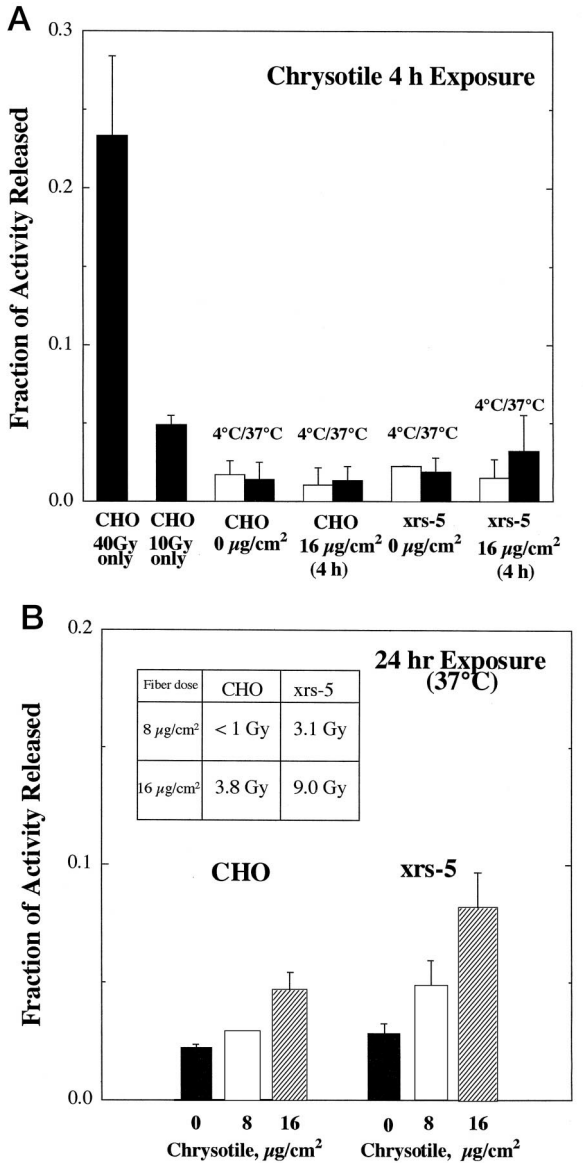

Fig. 3. *A*, measurement of DNA DSBs in CHO and xrs-5 cells exposed to high doses of chrysotile fibers for 4 h at 4°C and 37°C, as measured by CHEF gel electrophoresis. For comparison, data for radiation (γ-rays)-induced DNA DSBs in CHO cells are given. The results were obtained from two to three independent experiments. *B*, induction of DNA DSBs in CHO and xrs-5 cells exposed to chrysotile for 24 h at 37°C, as measured by CHEF gel electrophoresis. Radiation-equivalent amounts of DNA DSBs are provided for reference (chrysotile background values were subtracted beforehand for these calculations). These results were obtained from three independent experiments.

experiments, where the rate of decrease in cell number as a function of chrysotile doses as compared with nontreated control in CHO and xrs-5 cells are given. The data indicate that xrs-5 cells manifested higher growth delay than CHO cells. The growth delay data in Fig. 2 seems to reflect the cell survival data for 24 h in Fig. 1. These results on cell growth again demonstrate that xrs-5 cells are more sensitive to asbestos than CHO cells. Interestingly, xrs-5 cells showed a significantly longer cell cycle delay in response to ionizing radiation as compared with CHO cells (19).

Because the xrs-5 cell line is deficient in DNA DSB repair, we examined DNA DSB status in xrs-5 and CHO cells exposed to chrysotile under various experimental conditions using CHEF gel electrophoresis. Fig. 3*A* gives results for a short-term exposure at 4°C and 37°C temperatures in these cell lines. The reason for taking the 4°C measurement in addition to 37°C is to study the repair factor as

cells repair machinery is active at 37°C, but not active at 4°C. In none of the cases, no increase in DNA DSBs was observed in CHO cells, as well as xrs-5 cells, exposed to a very high concentration (16 $\mu g/cm^2$) of chrysotile. Studies with lower concentrations of chrysotile gave similar results as in Fig. 3*A* (data not shown). For comparison, the level of DNA DSBs produced by ionizing radiation was given in the figure. In these radiation DSB measurements, background levels (generally 0.01–0.03 in FAR) have been subtracted.

In contrast to 4-h DSB measurement, significant increases in DSBs were observed mainly in xrs-5 cells exposed to chrysotile for 24 h (Fig. 3*B*). Dose-dependent rise in DSB response was seen in both cell lines; however, xrs-5 cells gave a more pronounced DNA DSB yield in 8 and 16 $\mu g/cm^2$ exposure. The statistical evaluation indicates that the mean values between 0 and 8 $\mu g/cm^2$, as well as 16 $\mu g/cm^2$, are significant in xrs-5 cells ($P < 0.05$), and the mean values between 0 and 16 $\mu g/cm^2$ are significant in CHO samples ($P < 0.05$). The radiation-equivalent values for these chrysotile DNA DSBs are given in the inset of Fig. 3*B*. These values were calculated from a fitted curve for the γ-ray dose-response curve. This sensitivity to DNA DSBs by chrysotile in xrs-5 cells could be attributed to the DNA DSB repair defect in this mutant cell line. The yield for DNA DSBs without any repair may be identical for both cell lines, but xrs-5 cells cannot rejoin some of their DSB efficiently, thus giving the higher yield. The identical initial DNA DSB dose response by X-rays has previously been reported in these two cell lines (20). These results also indicate that a longer period of exposure is necessary for induction of DNA DSBs in asbestos-treated cells.

In this study, we have shown for the first time that an ionizing radiation-sensitive cell line is also sensitive to asbestos exposure. Using this DNA DSB-deficient cell line, we also demonstrated that asbestos fibers can produce DNA DSBs in prolonged exposure (24 h). Our data suggest that additional studies are needed to clarify the relationship between asbestos injury and DNA DSBs, which could lead to a carcinogenic process if left unrepaired. The chrysotile doses we used for our DNA DSB studies were estimated to be about $2–4 \times 10^9$ fibers/gm of medium. This dose range does not deviate significantly from the measured asbestos doses ($\sim 10^7–3 \times 10^9$ fibers/gm of dry tissue) of the dry lungs of the asbestos workers who had mesothelioma or lung cancer (21, 22). Thus, our system using xrs-5 cells might be useful in estimating the level of DNA damage induced by asbestos in an *in vitro* setting.

A simple explanation for the increased sensitivity of xrs-5 cells to asbestos would be the production of higher number of DNA DSBs in this mutant as clearly shown in Fig. 3*B*. However, the mechanism of fiber-induced DNA DSB may be different from that of radiation origin. As can be observed in Fig. 3*A*, there are no significant DNA DSBs produced at a short time exposure (4 h), but a longer time period gave substantial DNA DSBs, particularly in xrs-5 cells. It is probable that free radicals generated by asbestos keep stimulating DNA and terminate in DNA DSBs after a certain time period. This phenomenon is more pronounced in DNA DSB repair-deficient cells, as demonstrated in xrs-5 cells. Because Ku 80 is suggested to mediate the xrs phenotype, it is likely that this protein is also involved in repairing the DNA damage induced by asbestos fibers.

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Cancer Res 1999;59:298-300.