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DNA strand breaks, repair, and survival in x-irradiated mammalian cells

(reproductive death/unrepairable strand breaks/density gradient sedimentation/Chadwick-Leenhouts model)

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ABSTRACT **The yields of unrepairable single- and double-strand breaks in the DNA of x-irradiated Chinese hamster cells were measured by low-speed neutral and alkaline sucrose density gradient sedimentation in order to investigate the relation between these lesions and reproductive death. After maximal single-strand rejoining, at all doses, the number of residual single-strand breaks was twice the number of residual double-strand breaks. Both double-strand and unrepairable single-strand breaks were proportional to the square of absorbed dose, in the range 10–50 krad. No rejoining of double-strand breaks was observed. These observations suggest that, in mammalian cells, most double-strand breaks are not repairable, while all single-strand breaks are repaired except those that are sufficiently close on complementary strands to constitute double-strand breaks. Comparison with cell survival measurements at much lower doses suggests that loss of reproductive capacity corresponds to induction of approximately one double-strand break.**

The great majority, if not all, of the single-strand breaks (SSBs) in DNA of x-irradiated mammalian cells are repaired (1–19). It is unclear whether a few SSBs are not repairable. On the other hand, double-strand breaks (DSBs) are thought to occur in the proportion of one DSB for approximately 10 SSBs (20–24), and there is disagreement regarding the ability of mammalian cells to repair any DSBs (6, 8, 20–22, 25, 26).

The technical difficulties inherent in measurements of SSBs and, particularly, DSBs in mammalian DNA, using velocity sedimentation, have been to some degree resolved by many recent studies (e.g., refs. 11, 21, and 22). Nevertheless, the uncertainties regarding both unrepairable SSBs and repairability of DSBs are largely related to insufficient resolution and precision available previously from this technique. We report here measurements, of improved precision, that may contribute to resolving both uncertainties. We discuss, also, implications for microdosimetric arguments, which have been advanced to show that DSBs cannot be the major lethal lesion in mammalian cells.

MATERIALS AND METHODS

Chinese hamster cells (V79-379-A) were grown for 16–18 hr on 30 mm glass petri dishes containing 2.5 ml of Eagle's minimal essential medium with Earle's salts and 10% fetal calf serum supplemented with 0.5 μCi/ml of [^{3}H]dThy. Irradiations were performed at 0° in air with a Westinghouse therapy x-ray unit, operated at 250 kV and 15 mA with 1 mm Al additional filtration, which delivered a dose rate (corrected for backscatter) of 1350 rad/min. The medium was replaced before reincubation at 37° in 5% CO_2 atmosphere. Cells were recovered by scraping into phosphate-buffered saline.

Cell aliquots (10^5 cells per ml) were lysed for 2 hr at 20° on 5–20% sucrose density gradients in a Spinco SW 50.1 rotor. Saline-EDTA (0.15 M NaCl, 0.10 M EDTA) was the solvent for the gradient solutions, which differed by the presence of 0.2 M NaOH (pH 12) or 0.05 M Tris buffer (pH 8.5). SSBs were assayed on pH 12 gradients sequentially loaded with 100-μl layers of 1 M NaOH, cells, and H_2O, while DSBs were monitored on pH 8.5 gradients overlaid with 50-μl aliquots of cells, 1 mg/ml of nuclease-free Pronase (Calbiochem), and 0.1% sodium dodecyl sulfate. Native DNA was normally sedimented for 920 min at 10,000 rpm and denatured DNA for 105 min at 30,000 rpm.

The sedimentation velocity relation for determining DNA molecular weight was calibrated using intact strands of *Bacillus subtilis* bacteriophage SP50 DNA (duplex molecular weight 10^8). DNA strand breaks were quantified using a computer simulation of random breakage (27) applied to the molecular weight distributions from control samples (irradiated to 10.8 krad and incubated, see below).

Abbreviations: SSB, single-strand break; DSB, double-strand break.

RESULTS AND DISCUSSION

Absence of double-strand rejoining

Three pairs of sedimentation profiles are shown in Fig. 1; each pair consists of profiles from an alkaline and a neutral gradient sedimentation of cells scraped from the same dish. The three pairs are controls (see below), cells irradiated to 54 krad and held at 0° until lysis, and cells incubated for 210 min at 37° after 54 krad. The latter case represents a separate experiment, shown here for convenient comparison, which is possible because the positions of the profiles are reproducible between experiments. The lines shown are the best fit, to profiles for irradiated cells, from computer simulation of random breakage applied to control DNA (27). The close agreement between lines and data points indicates that profiles for irradiated cells correspond to random breakage products. That is, both single- and double-strand breakage, and single-strand rejoining appear to be random (28).

The profiles from irradiated cells show that, although 95% of the single-strand breaks were repaired, neither the number nor the distribution of double-strand breaks was significantly different after incubation.

Nature of unrepairable single-strand breaks

In the above experiment, centrifugation times were such that molecular weights from the neutral and alkaline gradients were closely in two to one correspondence for any sedimentation distance in the central region of the gradients (see upper and lower scales, Fig. 1). It is thus evident that the single-strand profile from incubated cells corresponds accurately to halves of the double-strand fragments from either incubated or unincubated cells. This observation was confirmed by a more detailed comparison. Thus, after incuba-

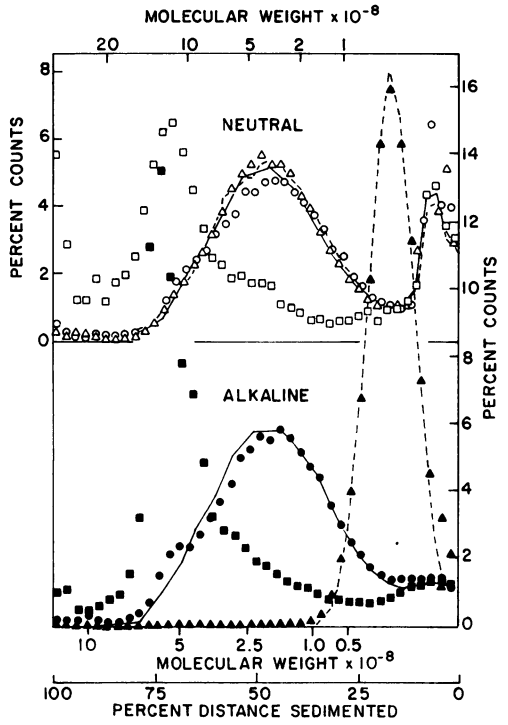

FIG. 1. Sedimentation profiles (points) from neutral (open symbols) and alkaline (filled symbols) sucrose gradients compared with best fit profiles from computer simulation of random breakage (lines). Pairs of profiles shown are from controls irradiated to 10.8 krad and incubated for 210 min at 37° (squares), cells irradiated to 54 krad with no subsequent incubation (triangles and dashed lines), and cells similarly irradiated and incubated for 210 min at 37° (circles and solid lines).

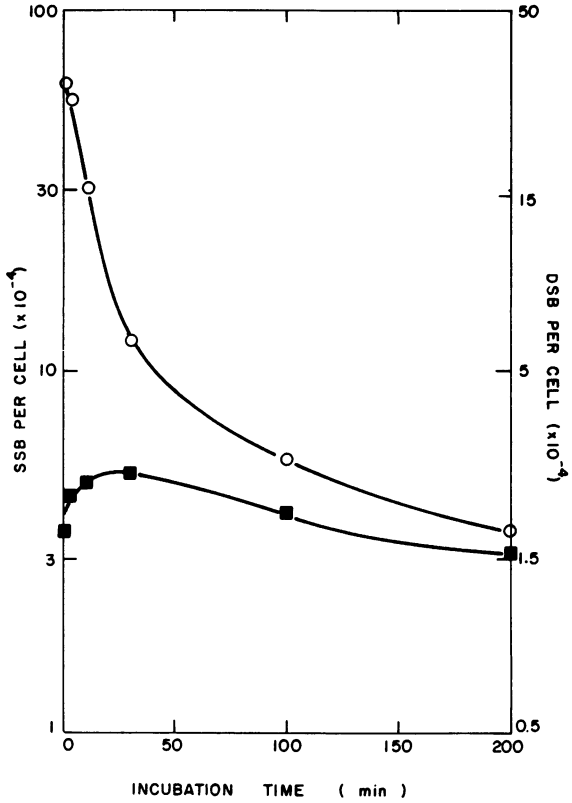

FIG. 2. Change in number of single- and double-strand breaks (left and right scales, circles and squares, respectively) with incubation time at 37°, after 54 krad of x-irradiation. The number of breaks per cell was determined by computer simulation of random strand breakage, and is based on 10^{-11} g of DNA per cell.

tion, there were two unrepairable single-strand breaks for each initial, or final, double-strand break.

The same result (not shown) was obtained with 310-min incubation. Using the computer simulation to quantify residual breaks, measurements of the kinetics of strand rejoining with incubation at 37° after irradiation to 54 krad (Fig. 2) also showed that any residual rejoining, after 200 min, was slow. The apparent increase in DSBs after a few minutes of incubation, and their disappearance, concomitantly with SSBs, was reproducible, and may be related to the shear artifact discussed below.

Thus we can detect, and quantify, SSBs that are unrepairable by the single-strand repair system, in the sense that any rejoining is more than an order of magnitude slower than the initial rate. That there are two such breaks for each DSB obviously suggests these SSBs are unrepairable because they pairwise constitute DSBs, and so the repair system lacks a substrate.

Dose dependence of residual breaks

Residual SSBs and DSBs were determined as above, after irradiation to various doses, and incubation at 37° for 210 or 310 min in two experiments. In each experiment SSBs and DSBs were again assayed from aliquots of the same cells. The number of breaks of both types, per G_1 phase cell, is shown in Fig. 3 as a function of the absorbed dose squared. At all doses, the number of unrepairable SSBs per cell was twice the number of DSBs, within the scatter of the points. The additional incubation time did not give rise to any systematic difference in residual breaks, further confirming the absence of detectable double-strand rejoining.

Sequential F-testing indicates that a linear dose dependence gives a highly significant lack of fit ($F = 54.45 \gg F_{3,15,0.99} = 5.42$), whereas a pure quadratic dose dependence yields a lack of fit that is not significant at the 1% level ($F = 4.31 < 5.42$). This fit would, of course be marginally improved by adding a (negative) linear or (positive) cubic term, but this would be imprudent with only five dose levels, especially since neither term corresponds to any known or postulated process. The least squares best fit to the pooled data versus dose squared yields a slope of 5.9 DSB/cell per krad^2. Additional support, for the validity of the quadratic relationship, accrues indirectly from the strict linearity observed for SSBs with the same alkaline gradient technique and a comparable degree of breakage, when cells are not incubated (28).

Controls used

When DNA from cells irradiated to less than 4 krad was sedimented on neutral gradients, much of the activity usually pelleted to the bottom of the tube, and the profile of the activity remaining in the gradient was irreproducible, as observed by other investigators (22). To avoid this problem, cells irradiated to 10.8 krad and incubated for 210 (or 310) min at 37° were used for controls (Fig. 1). The same procedure was followed for residual SSB measurements, though the problem does not arise in this case, in order to preserve the comparison with DSBs.

The question must be considered, whether this procedure has a detrimental influence on the data analysis. For two reasons we expect that it does not. Controls created in this way yield profiles on alkaline gradients that are indistin-

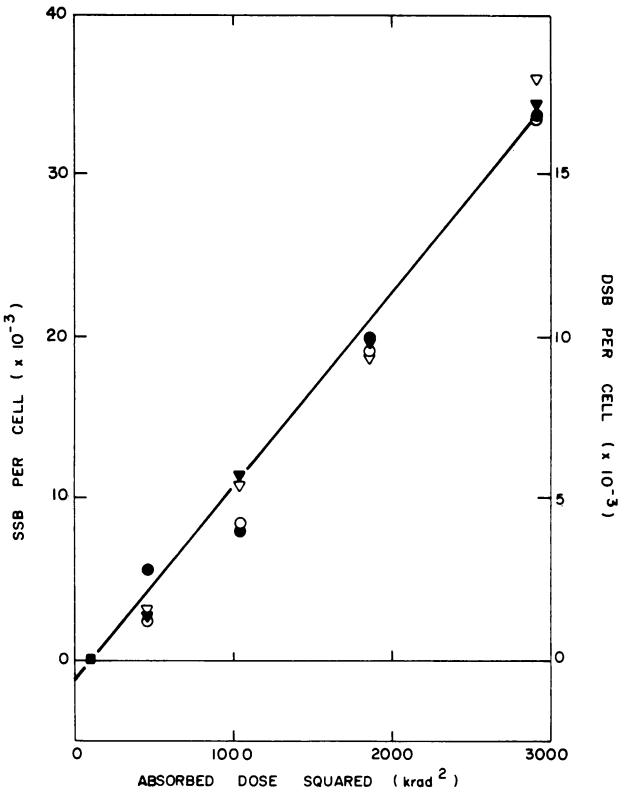

FIG. 3. Residual single- and double-strand breaks (filled and open symbols, left and right scales, respectively), from two experiments in which single-strand rejoining after irradiation proceeded to completion (incubation at 37° for 210 and 310 min, triangles and circles, respectively), plotted versus the square of absorbed dose. The filled square corresponds to the 10.8 krad controls (see *text*).

guishable from profiles for unirradiated cells, and we have found at higher doses that the alkaline gradient profiles correspond well with the neutral gradient profiles. Further, from Fig. 3 we expect 10.8 krad to yield only 0.3 DSB per molecule at the mode molecular weight (1.2×10^9; Fig. 1). Thus, while this dose is more than adequate, with the lysis and gradient conditions used, to disrupt or destabilize any complex or aggregate (22, 23), only an insignificant number of DSBs or unrepairable SSBs should be introduced.

Speed dependence

DNA of high molecular weight may sediment anomalously under sufficiently high *g*-forces, yielding a characteristically sharp profile, due to retardation of larger molecules (11). Generally, this anomaly was not observed with neutral gradients in the conditions used here, but, with alkaline gradients, retardation effects appeared for molecular weights greater than 6×10^8, at 30,000 rpm. Detailed comparisons between single- and double-strand profile shapes (Fig. 1) from the present experiments show a distorted leading edge on the low dose, and particularly the control, profiles consistent with retardation above 6×10^8 molecular weight. This small degree of retardation would introduce an error in the number of SSBs, at each dose, of order 500 breaks per cell, which may be neglected.

Possible biological significance

DSBs in mammalian cells have previously been considered to be induced in proportion to absorbed dose (20–24). This has not, however, been based on a detailed examination of whether the data would support this or other interpretations. The scatter of data in these studies is generally much larger than that reported here, so that statistical tests cannot distinguish between linear and quadratic dose dependences, and the dose range is typically an order of magnitude higher. The high dose rates used give rise to the question whether radiolytic oxygen consumption may have reduced the strand breakage at high doses. In only one case (21) have the profiles been shown to correspond to random breakage, and in that case only over parts of the profiles. As well, it is possible that, in these studies, some DSBs are produced in high molecular weight DNA by shear, during lysis or sedimentation, at SSB sites. Such DSBs could appear to repair due to removal of shear sensitive sites by single-strand rejoining. Recent data (23) have suggested that DSBs, in mammalian cells incubated after irradiation, may not depend linearly on dose. Thus, previous studies do not establish definitively the dose dependence of DSBs in the dose range below 50 krad.

Our finding, that DSBs are induced in proportion to the square of absorbed dose in the range 10–50 krad, is based on profiles that correspond well to random breakage, and is reinforced by the two-to-one ratio of unrepairable SSBs to DSBs measured in different gradient systems. This finding has considerable interest for both mammalian cell biology and microdosimetry.

While some direct relationship between strand breaks and mammalian cell survival is widely anticipated (8), and has been supported by some studies (26, 29, 30), other evidence (13, 14, 16, 18, 19, 31) has been interpreted as inconsistent with, or at least not favoring, any simple causative relation. A model of eukaryotic cell survival, proposed by Chadwick and Leenhouts (32), specifies that unrepaired DSBs are the lethal lesion. They may result either from single events, whereby one ionizing particle causes breaks in both DNA strands, or from double events, which require two adjacent but statistically independent SSBs in complementary strands. The model thus represents the surviving fraction of cells, after absorbed dose D, as

$$S/S_0 \doteq \exp(-\alpha D - \beta D^2) \quad [1]$$

where α and β are the coefficients for lethal events produced by one and two particles, respectively (see ref. 32 for a fuller discussion). This equation has been shown to describe accurately the survival of Chinese hamster fibroblasts at all stages of the cell cycle (33), and in exponential growth with or without a variety of radiosensitizing and protecting agents (34). It follows from the model that unrepaired DSBs in the same cells are given by

$$B = (\alpha D + \beta D^2)/p \quad [2]$$

where p is the probability that an unrepaired DSB will cause reproductive death. The quadratic term will dominate for doses greater than α/β (about 800 rad for G_1 phase cells; ref. 33), and thus the model would predict that the linear component of DSBs should be unobservable in the dose range of experiments reported here.

Since we do not detect DSB repair, and assuming that the quadratic component of DSB induction at lower doses is similar to that measured above 10 krad, we can estimate p for this model by the ratio of β from survival (4.0×10^{-6} rad^{-2} for G_1 phase V79 cells; ref. 33 and unpublished data) to the slope of the line in Fig. 3 (5.9×10^{-6} rad^{-2} based on

10^{-11} g of DNA per cell in G_1 phase; ref. 35 and unpublished data) which yields $p = 0.7$. Since the two values, in the quotient, are derived from quite different techniques, involving different sources of error, this estimate may not be significantly less than one.

From microdosimetric considerations it has been concluded that an α/β value, from survival data, of order 800 rad, implies that damage is deposited in a target with diameter two orders of magnitude greater than the diameter of duplex DNA (36, 37). Alternatively stated, in terms of the Chadwick-Leenhouts model, DSBs from two particle events (induced in proportion to the square of absorbed dose) should be insignificantly few for doses less than about 10^5 rad, if SSBs must closely coincide [within <10 base pairs in solution (38)] to form a DSB. However, the results reported here indicate that the measured coefficient, for double-strand breakage by two particle events, is not only sufficiently large to support the assumptions of the Chadwick-Leenhouts model, but is suggestive of a closely one-to-one relation between DSBs and cell death. If this result is confirmed, reasons must be sought for the apparent discrepancy with microdosimetric theory.

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