EFFECT OF DIFFERENTIATION ON THE REPAIR OF DNA SINGLE STRAND BREAKS IN NEUROBLASTOMA CELLS

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Summary: The capacity of murine C-1300 neuroblastoma cells to repair x-ray induced DNA single strand breaks was compared under two distinct culture conditions. Growing undifferentiated cells maintained in normal medium containing 10% fetal calf serum showed substantial break rejoining during a 30 minute repair period. Non-growing, differentiated cells in serum-free medium did not appear to be capable of repairing such breaks. Whether this phenomenon is attributable to a loss of repair enzymes or to an increased lability of the differentiated cells related to adverse culture conditions is uncertain. The results suggest that (a) nuclear integrity is not absolutely vital to morphogenetic differentiation of C-1300 neuroblasts and (b) also imply that deterioration of tissue culture growth conditions may affect DNA repair mechanisms in a fashion independent of other complex biosynthetic events.

The repair of x-irradiation damage by non-proliferating and/or aging mammalian cells is of interest since both normal and malignant tissues contain significant numbers of non-cycling cells. Permanently post-mitotic cells such as neurons are of particular interest since non-repaired genetic damage could have long term physiological importance (1, 2).

An indirect attempt to evaluate this phenomenon in vitro was first made by Epstein et al. who reported that fibroblasts from patients with progeria (a genetic disease hypothesized to represent premature aging), had a diminished capacity to repair x-ray induced DNA strand breaks (3). More recent experiments have suggested that this phenomenon may be due to a particular lability of this type of cell to tissue culture manipulation (4, 5).

The development of the C-1300 murine neuroblastoma system (6, 7) which differentiates when serum is removed from the culture medium (8) offered a unique

opportunity to examine radiation repair in another type of cell system characterized by impaired proliferation but which on the other hand is known to be capable of other significant biosynthetic events including axon formation (6-9), neurotransmitter synthesis (10) coupled to physiological development such as action potentials etc. (11, 12).

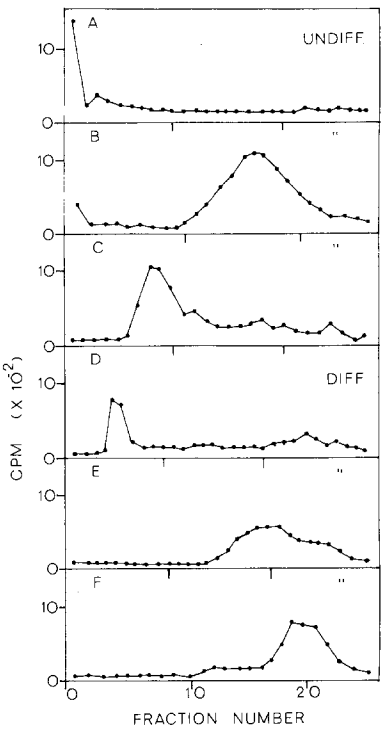
Our results indicate that there is an almost complete loss of the capacity to repair x-ray induced strand breaks in differentiated neuroblastoma cells, under conditions in which maintenance of these other physiological activities is known to be maintained (cf. ref. 9). Whether this loss is related to the phenomenon of differentiation *per se* or is an artifact of the adverse culture conditions required to induce differentiation is not known but we prefer the latter explanation. The results would appear pertinent to the questions raised in the progeria studies (3-5).

MATERIALS AND METHODS

Murine C-1300 cells were maintained in exponential growth in modified Dulbecco's medium as described in detail elsewhere (9). To compare the ability of dividing (undifferentiated) to non-dividing (differentiating) neuroblasts to repair DNA single strand breaks, the growing cells were first labelled with ^{3}H-thymidine for one doubling period and the culture then sub-divided into 6 parts. In each experiment, three of the sub-cultures were immediately studied for single strand break rejoining. The others were plated out overnight to allow attachment, the complete medium removed and replaced with serum-free medium, and the cells allowed to differentiate. (The details of the subsequent morphological changes have been described in detail by Seeds et al. (8) and were reproduced identically by our studies.) After three days had elapsed and axon formation was well-established, the differentiated cultures were assayed for single strand break rejoining. The alkaline sucrose gradient method of McBurney et al. (13) was used throughout these experiments. Each assay included a non-irradiated culture, a culture taken immediately following irradiation and a third irradiated culture incubated at 37^{o} for 60 minutes prior to gradient analysis. Procedural details are given in the legend to the figure. Identical results were obtained in three separate experiments performed in different weeks.

RESULTS

When exponentially growing mouse neuroblastoma cells are subjected to heavy (10 krad) irradiation (panels A and B in Figure) there is an immediate shift in the DNA sedimentation pattern towards the top of the gradient consistent with multiple single strand DNA breaks. The pattern is restored partially towards normal if the cells are allowed to incubate at 37^{o} during the post-radiation recovery period (panel C). The results do not appear to differ significantly



Legend: Effect of differentiation on repair of DNA single strand breaks in murine C-1300 neuroblastoma cells. Cells in log growth were labelled for 24 hours with ^{3}H thymidine (0.1 μCi/ml, 18 Ci/mM). The culture was then divided into 2 subcultures. One subculture (non-differentiated) was evaluated immediately for the rejoining of x-ray induced strand breaks while the other was washed once in Hank's solution and plated out in serum-free medium for 3 days to induce differentiation. Each subculture was plated into 3 equal portions. The control cultures (non-differentiated) were plated in complete medium and allowed to attach for 4 hours prior to study. Each set of cultures was evaluated for DNA sedimentation in alkaline sucrose gradients following either no radiation (panels A and D), 10 krads radiation, with no repair (panels B and E), or 60 minutes post-radiation repair (panels C and F). Irradiation was performed in situ in all cases using Cobalt-60 gamma rays. To evaluate the sedimentation of the pre-labelled DNA each culture was trypsinized (10 minutes at 37°) and resuspended in Tris buffer (pH 7.4). Approximately 0.05 ml of the cell suspension containing 5-10x10^{4} cells was then placed on top of a layer of 2 ml of 2% sucrose in water which had itself just been layered on a pre-formed 10% to 30% alkaline sucrose gradient made up in 0.3N NaOH, 0.01 M EDTA, and 0.5 M NaCl (total of 30 ml in a Beckman SW 25.1 rotor nitrocellulose tube). Each gradient was pre-equilibrated to 4°C temperature for several hours prior to use. The loaded gradients were then stored at 4°C for 16-20 hours prior to centrifugation to allow DNA release and denaturation. Centrifugation was at 23,500 rpm at 4°C for 4 hours. Each gradient was then fractionated by puncturing the bottom of the tube and collecting 1.0 ml fractions. To each fraction was added 5.0 ml of 5% trichloroacetic acid (TCA) and the nucleic acids allowed to precipitate for 30 minutes at 0°C. The precipitates were then collected on GF/C glass fiber filters, washed twice with cold 5% TCA and 95% ethanol, dried overnight and then counted in a Beckman CPM 200 liquid scintillation counter. All cell incubations were at 37°. All sedimentation from right to left. Radiation induced a sharp shift from left to right in both undifferentiated (A-B) and differentiated cultures (D-E) consistent with DNA single strand breakage. This shift was partially restored in the non-differentiated culture (panel C) but not in the differentiated culture (panel F).

from our previously reported results with this method using cycling mouse leukemia cells (14). These shifts in the sedimentation pattern are consistent with the repair of radiation-induced DNA single strand breaks. In our hands sedimentation of non-irradiated DNA to the tube bottom frequently occurs when this lysis method is used, presumably representing still partially aggregated DNA in accord with the interpretations of McBurney et al. (15).

The pattern observed when differentiated neuroblastoma cells were studied was quite different. Sedimentation of non-irradiated samples showed a slight shift to the right when compared to the non-irradiated, undifferentiated control cells. Following 10 krads irradiation, the DNA again showed a shift in the sedimentation pattern towards lower molecular weight DNA. However, subsequent incubation at 37° for 60 minutes or longer had no effect on the pattern, suggesting that repair of single strand breaks does not take place under these particular conditions.

DISCUSSION

It has previously been shown that at least one enzyme activity related to DNA synthesis (thymidylate synthetase) is rapidly reduced when murine neuroblastoma cells stop dividing (16). If polynucleotide ligase activity (17) is responsible for the DNA single strand break rejoining seen here in the cycling cell form (panels A-C), then it might be postulated that under the starvation conditions we have employed, ligase activity is effectively lost leading to an inhibited DNA single strand break rejoining capacity. Adverse culture conditions such as serum-starvation may therefore affect DNA repair capacity as well as normal replicative and cell multiplication potential. The degree to which these two effects may affect post-radiation cell survival is probably complex. For example, the data of Belli and Shelton indicated that a brief post-irradiation incubation in a balanced salt solution at 37° had a protective effect (increased survival) while more prolonged incubation (over 2 hours) was deleterious with a subsequent progressive decline in survival to substantially less than control values (18). The cells studied here most closely resemble the latter conditions.

These experiments are of interest primarily because it is well-established that elaborate physiologic changes such as axon formation and related events can occur in this type of cell despite an apparent degeneration in at least one component of the DNA maintenance mechanisms. The recent demonstration that enucleated neuroblasts can form axons similarly illustrates the independence of such functions (19).

There is a slight suggestion that the sedimentation of non-irradiated differentiated neuroblastoma DNA is less in tissue culture (panel C versus A in the Figure). Whether this observation has any bearing on the elegant experiments by Wheeler and his colleagues on the DNA repair capacity of aging neurons (2, 20, 21) is questionable. Their most recent studies have shown that cerebellar neurons from both young and old beagles can partially repair DNA single strand breaks but the older animals show a progressive reduction in the size of control (non-irradiated) DNA (21). Similarly, since enucleated neuroblasts can also show significant morphogenetic changes (19) it seems unlikely there is any relation between the reported capacity of x-rays to induce differentiation in mouse neuroblasts (22) and the status of nuclear DNA other than the well-established ability of ionizing radiation to suppress DNA synthesis and cell-cycling. We have shown elsewhere that other cycle-active anti-metabolites can also induce neuronal differentiation (9).

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