SHORT COMMUNICATION

Radiation, heat and anti-melanin drug response of a transformed mouse embryo cell line with varying melanin content

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Repair of radiation damage as well as cellular and environmental factors can play a major role in radiosensitivity (Hall, 1978; Steel et al., 1983; Freeman et al., 1981). Both repair of sublethal damage and potentially lethal damage have been shown to increase cellular resistance to radiation (Elkind & Sutton, 1960; Philips & Tolmach, 1966; Little, 1969). The factors that influence radiosensitivity may play an important role in the treatment of tumour cells by radiotherapy. It is well known that melanoma tumours such as osteosarcoma, glioma and melanoma are difficult to control by radiotherapy and may be radioresistant (Habermalz & Fischer, 1976; Nilsson et al., 1980; Abe et al., 1979). Some earlier studies indicate that this resistance may be linked to the ability of cells to repair radiation damage (Weichselbaum et al., 1982; Weichselbaum & Little, 1983; Hahn & Little, 1972).

It was reported that melanoma was radioresistant and that survival curves demonstrated a large shoulder (Barranco et al., 1971; Smith et al., 1978; Trott et al., 1981). Several other reports indicated that melanomas responded better to fractionated radiotherapy if large fractions were given indicating a large SLD repair capacity. However, there is some debate regarding these findings (Hornsey, 1978; Overgaard, 1980; Trott et al., 1981).

In vitro studies of melanoma have been conducted with human cells and in such studies the normal parental cell strain was not available for comparison. In our laboratory, we have developed a melanoma-like cell line by transformation of the C3H-10T1/2 mouse embryo cell line developed by Reznikoff et al. (1973). This cell line (R25) possessed increased radioreistance in the survival curve shoulder region, produced melanin and contained melanosomes (Szekely et al., 1985). This cell line could be compared directly to its parental strain since it was readily available, whereas this is not possible with melanoma tumour derived cell lines.

The R25 cell line has been studied to address several questions arising from observations made in the study of melanoma tumours and melanoma cell lines. (1) Does the increased size of the survival curve shoulder indicate an increased capacity for radiation damage repair? (2) Does the presence of melanin influence radiosensitivity and heat sensitivity? (3) Is this melanoma cell line sensitive in its response to anti-melanoma chemical agents compared to its normal parental cell line and other transformants not exhibiting melanoma like properties.

The transformants were produced from the normal C3H-10T1/2 cells by radiation or H-ras oncogene transfection. The culture conditions, and transformation procedures have been previously described in detail (Raaphorst et al., 1985; 1987). Cells were cultured in a mixture of 1:1 Dulbecco's modified MEM and F12 medium containing 10% foetal calf serum. Details of cell culture, radiation procedures and experimental manipulation have been previously described (Raaphorst et al., 1985).

Table 1

| Cell type | G1 | S | G2+M |
|-----------|----|---|------|
| Normal    | 52.2 | 25.7 | 22.1 |
| R25       | 53.3 | 24.6 | 22.1 |

Data fitted by polynomial fitting algorithms supplied by Ortho. Maximum error is <10%. Flow cytometry was done by isolating nuclei and staining with ethidium bromide as previously described (Raaphorst et al., 1985).
Figure 1 (a) Radiation survival curves of normal C3H 10T1/2 R19, CIRAS and R25 cells. The survival curve parameters are as follows: Normal, $\alpha = 0.39 \pm 0.05$; $\beta = 0.021 \pm 0.008$; $n = 2.0$; $D_0 = 1.52$; R25, $\alpha = 0.15 \pm 0.06$; $\beta = 0.045 \pm 0.009$; $n = 6.0$; $D_0 = 1.31$. The parameter for R19 and CIRAS are about the same as for the normal line. (b) The repair of sublethal radiation damage in normal (closed symbols) and R25 cells (open symbols) during incubation time at 37°C between two doses of radiation (indicated on abscissa) for plateau phase cells (circles) and exponential phase cells (squares). (c) The growth curves and melanin content of normal and R25 cells as a function of time in culture, • number of R25 cells per flask, ○ number of normal cells per flask, ■ melanin content of R25 cells and □ melanin content of normal cells. (d) Radiation and heat response of R25 cells isolated from spheroids with low (white), medium (brown) or high (black) melanin content. The parameters of the solid line on the radiation survival curve are $\alpha = 0.19 \pm 0.037$; $\beta = 0.063 \pm 0.009$; $n = 5.0$; $D_0 = 1.19$. The dashed curve represents response of normal cells from a monolayer.

| Presence of melanin | Cell line | PE | Percent survival |
|---------------------|-----------|----|-----------------|
|                     | C3H 10T1/2 |    | 4HA (6 x 10^{-4} M) | 2HA (3 x 10^{-3} M) |
| R19                 | 33 ± 3%    | 41 ± 3% | 41 ± 3% |
| R25                 | 26 ± 3%    | 43 ± 3% | 42 ± 3% |
| Ciras               | 21 ± 3%    | 31 ± 3% | 34 ± 3% |
| R25                 | 48 ± 3%    | 3.5 ± 1% | 9 ± 1% |

4HA, 4 hydroxyanisole; 2HA, 2 hydroxyanisole; PE, plating efficiency. Melanin was assayed as previously described (Szekely et al., 1985). HA treatment was given after overnight incubation.

At isosurvival levels a higher dose would be necessary for R25 possibly showing a greater degree of recovery.

Further tests were done to determine the nature of R25. Table II and Figure 1c show that R25 produced melanin while the normal and other transformed cell lines did not. The method for melanin analysis has been described in detail previously (Szekely et al., 1985). The melanin content of R25 cells increased as a function of incubation time in culture as shown in Figure 1c. Electron microscopy studies indicated the presence of melanosomes in these cells (Szekely et al., 1985).

The data in Table II show that R25 cells exhibited a greater response to 4-hydroxyanisole (4HA) and 2-hydroxyanisole (2HA) treatment than normal and the other X-ray transformed tumorigenic cell line R19 and the H-ras transfected tumorigenic cell line CIRAS 1. Cells were exposed for 2h to the 4HA and 2HA dissolved in culture medium. The latter two transformed cell lines exhibited the same response to 4-hydroxyanisole as the normal cell line. The increased response of R25 to 4HA and 2HA is similar to that of melanin producing melanoma tumours and cells (Riley, 1984; Meyskens, 1984). These data further indicate the melanoma nature of R25.

Figure 1d shows the heat and radiation response of R25 cells with various melanin content. When R25 cells were seeded into a 0.34% agarose medium they formed multicellular spheroids. In these cultures, spheroids of white, brown and black morphology developed after 10 to 16 weeks of growth. The number of spheroids that developed high
melanin content was dependent on the cell density and culture age and confirm the results of Weininger et al. (1978). Cells were obtained from black, brown, and white spheroids of R25 selected from culture after 10–14 weeks of incubation. Cells from these spheroids were isolated by trypsinization, assessed for melanin content, plated into flasks and tested for heat or radiosensitivity 16 h after plating. The responses to hyperthermia at 45°C and to radiation were the same for cells from the three types of spheroids. Also, no differences were observed for heating at 43.0°C (data not shown). Analysis of melanin content in the cells isolated from the black, brown and white spheroids indicated that cells from the black and brown spheroids contained 2–8 fold more melanin than cells from the white spheroids. Light microscopy study showed that cells from dark appearing spheroids contained high concentrations of melanosomes (Szekely et al., 1985).

Two studies showed that the addition of exogenous melanin in CHO cells or the variation of melanin content in B16 melanoma cells did not influence radiation sensitivity (Hopwood et al., 1985; Stephens et al., 1986). Our results on R25 confirm this finding and further indicate that melanin content over the range found in the white, brown and black spheroids also did not influence thermal sensitivity. However, the possibility cannot be ruled out that the range of melanin content studied was saturating for possible radiation effects down to the lowest level. For hyperthermia effects this possibility could be ruled out because the response of the normal cell line containing no melanin was about the same as that for R25.

Our data clearly indicate that the transformation of C3H-10T1/2 cells to R25 (melanoma like cell line) occurred concomitantly with an increase in the survival curve shoulder and an increased capacity to repair SLD compared to its progenitor. In addition, this cell line produced melanin and was sensitive to anti-melanin compounds while the other transformed cell lines were not. In a previous study it was shown that transformation led to random changes in radiosensitivity, primarily reflected in the survival curve D0 (Raaphorst et al., 1985). In R25, these changes were quite different in that radioresistance was reflected in the survival curve shoulder, typical of many human melanoma cell lines. This melanoma cell line and its normal progenitor cell line are being further investigated for differential responses to anti-melanin agents since these are already in use in the clinic (Webster et al., 1984; Morgan, 1984). These cell lines make a good system for the comparison of melanoma and normal cell responses to anti-cancer agents and treatments.

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