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CYTOTOXICITY OF RADIOSENSITIZERS IN MULTICELL SPHEROIDS: COMBINATION TREATMENT WITH HYPERTHERMIA

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Summary.—Spheroids of EMT6 tumour cells were grown in spinner culture. As with V79 cell spheroids, the combination of 1·5mM misonidazole and hyperthermia at 42·5°C for 1 or 2 h caused greater cytotoxicity than either heat or drug alone.

Radioresistant hypoxic cells at the centre of the spheroids were eliminated but other cells were also killed.

EFFECTIVE therapy of many solid tumours, including micrometastases, may be limited by the presence of noncycling, hypoxic cells which are relatively resistant to radiation and drugs. These cells may also exist in microenvironments where alterations in conditions such as pH and in concentrations of metabolites and nutrients have occurred. Since this combination of conditions is relatively unique in actively growing tumours, selective tumour therapy may result if drugs or treatment procedures which are activated in these microenvironments can be found.

In this regard, the cytotoxic properties of radiation sensitizing drugs (Sutherland, 1974; Hall and Roizin-Towle, 1975; Moore, Palcic and Skarsgard, 1976; Mohindra and Rauth, 1976; Sridhar, Koch and Sutherland, 1976; Brown, 1976; Foster *et al.*, 1976) and of hyperthermia (Gerweck, Gillette and Dewey, 1974; Hahn, 1974; Kim, Kim and Hahn, 1975; Overgaard, 1975) are of special interest. These agents can preferentially kill hypoxic cells as well as cells existing in acidic pH conditions. These two therapy modalities can also interact synergistically (Hall and Biaglow, 1977; Stratford and Adams, 1977; Sridhar and Sutherland, 1977; Bleehen, Honess and Morgan, 1977).

Chronically hypoxic cells and tumour-like microenvironments develop in multicell spheroids *in vitro* (Sutherland and Durand, 1976). Spheroids have been used as a tumour model system to assess the influence of hyperthermia and sensitizers on the radiation response of cells (Durand, 1977; Sutherland and Durand, 1972; Sutherland and Richardson, 1974; Sutherland *et al.*, 1978) and the modification of oxygenation status due to effects on respiration (Biaglow and Durand, 1976; Durand and Biaglow, 1974; Sutherland, 1975*b*). Cells in the central regions of spheroids were also shown to be selectively killed by each of these agents, when administered separately (Sutherland, 1975*a*; Sutherland, 1974; Sridhar and Sutherland, 1977). Recently we have demonstrated a significant positive interaction when hyperthermia and the nitroimidazole radiosensitizer Ro-07-0582 (misonidazole) are used together (Sutherland and Sridhar, 1978; Sridhar and Sutherland, 1977). The experiments reported here are an extension of those studies with misonidazole and other nitroimidazoles.

METHODS

Multicell spheroids were grown in suspension in spinner flasks in Eagle's basal medium (BME) with 5% foetal calf serum (FCS) for Chinese hamster V79-171b lung cells and 15% FCS for EMT6 mammary tumour cells

(Sutherland and Durand, 1976). Spheroids used in different experiments ranged from 480 to 850 μm in mean diameter and contained necrotic centres and hypoxic cells, surrounded by a zone of noncycling or poorly cycling cells.

Spheroids were held in complete medium in spinner flasks in water baths at temperatures of 41–43°C with or without 1·5 mM sensitizers for different treatment periods up to 3 h. This drug concentration was chosen since it represents the upper limit of concentration which can be attained with misonidazole in humans (Gray *et al.*, 1976). After each treatment period a known number of spheroids (usually 15–20) was dissociated to a single cell suspension by gentle trypsinization (0·25% trypsin for 12 min). The cells were counted with an electronic particle counter, diluted and plated for colony formation assay after 9 days of incubation for V79 cells and 13 days for EMT6 cells. The fraction of viable clonogenic cells was multiplied by the total cells per spheroid to give the total clonogenic cells per spheroid.

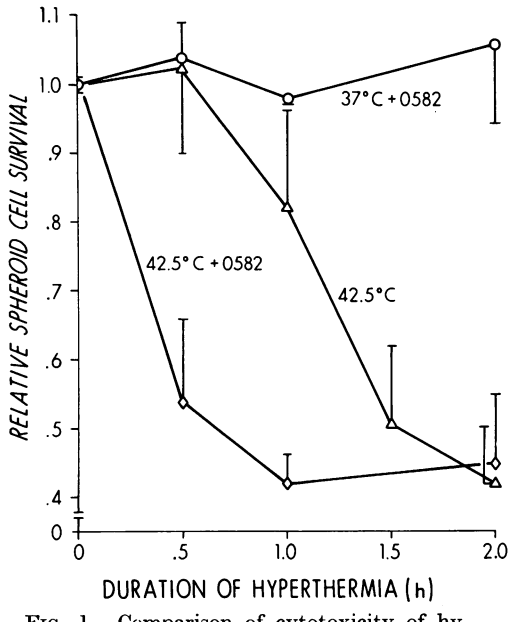

FIG. 1.—Comparison of cytotoxicity of hyperthermia and Ro-07-0582 (misonidazole) plus hyperthermia in EMT6 spheroids. Cell survival is expressed relative to spheroids incubated at 37°C. Average of 3 separate experiments. Misonidazole concentration = 1·5 mM. Spheroid diameter = 854 ± 60 mM. Errors = ± 1 s.d.

RESULTS

Our previous results with V79 cell spheroids showed that when 1·5 mM misonidazole and hyperthermia were used together there was significantly greater cytotoxicity than with either heat or drug alone (Sutherland and Sridhar, 1978; Sutherland *et al.*, 1978). The number of clonogenic cells per spheroid decreased by about 50% compared with heat alone, when misonidazole was combined with hyperthermia at 42 and 43°C for 1 and 0·5 h respectively. In the presence of the drug, shorter hyperthermic treatments were required to produce amounts of cytotoxicity similar to heat alone. Alternatively, the time required for the drug to produce similar levels of cytotoxicity (30 to 50%) was lowered from about 3 days at 37°C (Sridhar, Koch and Sutherland, 1976) to 90 min at 42°C. There was a plateau in cell survival at 42°C in the presence of the sensitizer after heat treatment of longer than 90 min suggesting some selection for cells within the spheroids which are particularly sensitive to the combined treatment.

Similar experiments were performed with EMT6 mammary tumour spheroids to determine the kinetics of this cytotoxicity (Fig. 1). Compared with hyperthermia alone at 42·5°C there was a significant increase in cytotoxicity at all treatment periods up to 2 h when 1·5 mM misonidazole was combined with hyperthermia. No cytotoxicity was observed at 37°C during these experiments. Hyperthermia alone produced a progressive decrease in cell survival beginning at 0·5–1·0 h. Almost half of the cells in the spheroids were killed by the combined treatment in only 0·5 h, while no cytotoxicity occurred at this time with hyperthermia alone. A plateau occurred after 1 h as was seen previously with V79 spheroids using sensitizers plus hyperthermia (Sutherland and Sridhar, 1978; Sridhar and Sutherland, 1977), or with

sensitizers alone after several days at 37°C (Sutherland, 1974; Sridhar, Koch and Sutherland, 1976).

On the basis of these and previous results a series of experiments was performed using both V79 and EMT6 spheroids heated to 42·5°C for 1 or 2 h in the presence or absence of 1·5 mM misonidazole (Table). Both types of cells in spheroids responded similarly to the different treatments. The cytotoxicity produced at 42·5°C for 1 h was significantly greater when heat treatment was combined with the drug. Although there is some cell death when the drug is present at 37°C, this does not increase at 2 h and previous studies have shown that levels of cytotoxicity seen here at 42·5°C in the presence of the drug (35–45%) do not occur until after several days' continuous drug exposure at 37°C (Sridhar, Koch and Sutherland, 1976). These results also show that there is little difference in the cytotoxicity obtained by increasing the hyperthermic treatment from 1 h to 2 h in the presence of the drug. This would be expected if there is a particularly sensitive subpopulation of cells, as suggested by the plateau in the curve for 42·5°C plus misonidazole (Fig. 1).

TABLE.—*Effect of Hyperthermia plus Ro-07-0582 on Clonogenic Capacity of Spheroid Cells*

		Relative† clonogenic cells/spheroid (%)	
V79	Spheroids	1 h	2 h
	37°	100 (8)	100 (8)
	37°+ 0582*	91·7 ± 6·8 (7)	92·9 ± 5·4 (3)
	42·5°	90·3 ± 1·6 (8)	46·7 (1)
	42·5°+0582	66·8 ± 4·8 (7)	58·4 ± 12·0 (5)
EMT6	Spheroids		
	37°	100 (6)	100 (6)
	37°+0582	91·4 ± 6·1 (5)	94·4 ± 8·3 (6)
	42·5°	81·1 ± 6·5 (5)	55·0 ± 6·6 (6)
	42·5°+0582	55·3 ± 6·4 (6)	52·7 ± 5·5 (6)

* Ro-07-0582 concentration = 1·5 mM

† Cell survival is expressed relative to spheroids incubated at 37°C; errors = ± 1 s.e.

Numbers in brackets refer to number of separate experiments.

Several 2- and 5-nitroimidazole analogues were evaluated in different experiments (Fig. 2). Although not significantly different, it appears that the two 5-nitroimidazoles studied may be more effective than the 2-nitroimidazoles when combined with hyperthermia. About twice as many cells were killed by Ro-05-0207 (ornidazole) than by misonidazole. No cytotoxicity was found at 37°C with any of these sensitizers.

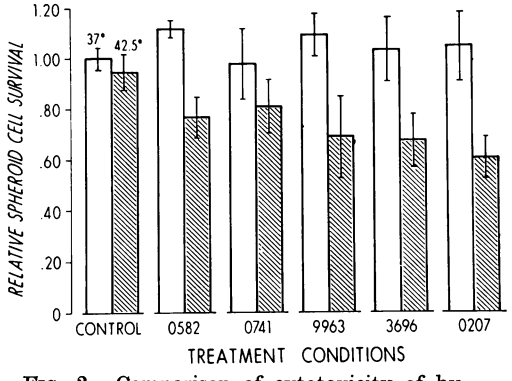

FIG. 2.—Comparison of cytotoxicity of hyperthermia with and without different nitroimidazole radiation sensitizers in EMT6 spheroids. All treatments were for 1 h. Cell survival is expressed relative to spheroids incubated at 37°C. Concentration of sensitizers = 1·5 mM. Sensitizers used: Ro-07-0582 (misonidazole) = 3-methoxy-1-(2-nitro-1-imidazolyl)-2-propanol; Ro-07-0741 = 3-fluoro-1-(2-nitro-1-imidazolyl)-2-propanol; Ro-05-9963 = 3-(2-nitro-1-imidazolyl)-1,2-propanediol; Ro-07-0207 = 1-(3-methoxy-2-hydroxypropyl)-2-methyl-5-nitroimidazole; Ro-11-3696 = 1-(3-methoxy-2-hydroxypropyl)-2-methyl-5-nitroimidazole. Errors = ± 1 s.d.

In order to determine the effect of the combined treatment on the hypoxic fraction of cells, the spheroids were irradiated to obtain survival curves (Fig. 3). Spheroids were pretreated with 1·5 mM misonidazole for 1 h at 42·5°C and then irradiated at 37°C 15 min after washing out the drug. Approximately 30% of the cells were killed by the combined treatment alone in the absence of irradiation, as found in other experiments reported here. There was a marked increase in cytotoxicity after irradiation; the surviving fraction was much less at all radiation doses compared with spheroids that had been

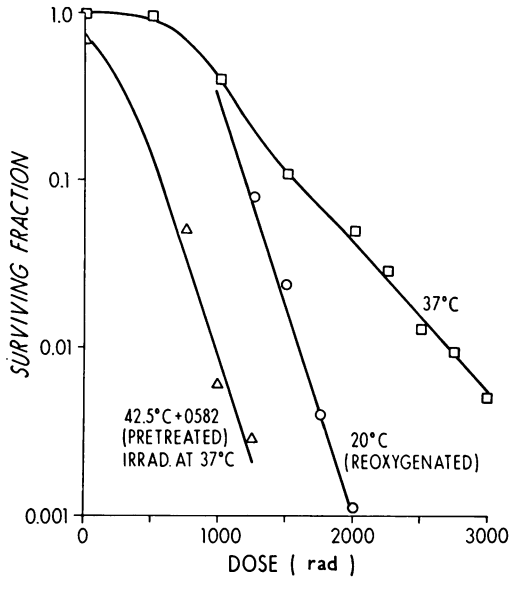

FIG. 3.—Radiation survival of cells in Chinese hamster V79 spheroids after pretreatment for 1 h with 1·5 mM misonidazole plus 42·5°C. Spheroids were grown and irradiated in spinner flasks in 5% O_2. Irradiation at 37°C began 15 min after washing out the drug.

reoxygenated after inhibition of respiration at 20°C. By comparison of the slopes of the curves it is apparent that pretreatment with hyperthermia plus misonidazole eliminated the resistant hypoxic cells. The total dose reduction factor at surviving fractions below 0·1 was 2·8, compared with 1·6 for reoxygenated spheroids. The oxygen enhancement ratios based on D_0 values were 3·0 for both reoxygenated spheroids and spheroids pretreated with hyperthermia plus misonidazole.

DISCUSSION

These results show that a combination of moderate hyperthermia and low concentrations of nitroimidazole radiation sensitizers can interact positively to kill up to 50% of the cells in multicell spheroids. This interaction may have several potentially significant therapeutic advantages. The duration of hyperthermia required to produce a given level of cytotoxicity is reduced by the presence of sensitizers, *e.g.* by a factor of 3 at the 0·55 survival level (Fig. 1). By combining hyperthermia with sensitizers it is possible to obtain similar effects at lower temperatures. Alternatively, the total amount of sensitizer required to reduce cell survival by 30–50% is greatly reduced by combination with hyperthermia because this level of cytotoxicity can only be obtained after several days of continuous drug treatment at 37°C (Sridhar, Koch and Sutherland, 1976).

Further experiments are required to assess the degree of selectivity of the cytotoxicity produced by the combined treatment for periods less than 2 h. Certainly cells other than those which are radiobiologically hypoxic are being killed. Histological observations of spheroids treated in these and other experiments (Sutherland, 1974) suggest, however, that the predominant cells which are being killed are those which are centrally located in the spheroids. Those would be somewhat hypoxic and may be sensitive because of the microenvironment in this area. On the other hand, the possibility that aerobic cells in normal tissues may be damaged by this combined therapy must be carefully evaluated to determine temperatures, drug concentrations and treatment periods which would produce maximum therapeutic effectiveness.

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