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THERMAL ENHANCEMENT OF CELLULAR RADIATION DAMAGE: A REVIEW OF COMPLEMENTARY AND SYNERGISTIC EFFECTS

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Abstract

Hyperthermia treatment can kill mammalian cells in a time and temperature dependent manner. Thermal sensitivity varies extensively among various cell lines in culture and cellular molecular and ultrastructural studies have not resolved which cellular mechanisms underlie thermal cell killing and radiosensitization. The response of cells to heat and radiation are complementary under certain conditions found in human tumors, such as hypoxia, low pH, low nutrient and the S-phase of the cell cycle. Thus, hyperthermia can be used as a complementary treatment modality in the radiotherapy of human cancer. Further studies show that heat treatment causes radiosensitization which is in part associated with the inhibition of repair of radiation damage and is strongly dependent on temperature and on the sequencing. In addition, the conditions such as pH and oxygenation during treatment sequencing can influence the degree of recovery of cells. These factors may be exploited in optimizing therapeutic gain in clinical cancer therapy. Data are shown that transformation from the normal to the tumorigenic state causes random small changes in radiosensitivity and heat sensitivity. Also, treatments combining heat and radiation can lead to increased or decreased transformation in cells depending on the treatment sequence.

KEY WORDS: hyperthermia, radiation, radiosensitization, cell killing, cell transformation, radiation repair, thermotolerance, repair inhibition

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Introduction

It is well known that elevated temperatures (>40.0°C) can inhibit cell growth and cause cell death. This effect was indirectly noted in the historical use of heat in the form of cautery in cancer treatment as reported in the writings of Auleus Cornelius Celsus of Rome near the beginning of the first century (Shimkin 1977). Near the end of the 19th century, effects of elevated temperature in the form of fever were noted by Coley (1893) to cause tumor regression in cancer patients. Interest in hyperthermia as a cancer treatment agent grew until the discovery and implementation of radiation in cancer therapy. Since results from hyperthermia treatment were more variable than those from radiation treatment, hyperthermia research did not grow until its recent rediscovery as a cancer therapy agent. Since then, much research has yielded a wealth of information on hyperthermia effects in a wide range of biological systems (Dethlefsen & Dewey 1982, Overgaard 1984, Lowenthal 1984).

It was shown in animal studies and clinical trials that hyperthermia was primarily efficacious when applied in combination with radiotherapy (Dethlefsen & Dewey 1982, Overgaard 1984). These results indicated the possible complementary nature of heat and radiation treatments in human cancers. Research on the response to combined treatments of hyperthermia and radiation of mammalian cells cultured in vitro supported the clinical findings and were further directed at characterizing and optimizing combined treatment protocols in many mammalian cell lines (Dethlefsen & Dewey 1982, Overgaard 1984).

In this review, we shall deal with some aspects of thermal cell biology and its relationship to radiobiology and how in vitro results yield a rationale for

the use of hyperthermia in the treatment of cancer. In addition, we shall attempt to indicate areas where research on morphology and ultrastructure will yield further information regarding mechanisms of action. Emphasis will be placed on results from our research laboratory and how these relate to the results published in the literature.

Materials and Methods

Unless indicated otherwise, all cultured cells used in the experiments were grown in a 1:1 mixture of Dulbecco's MEM and Ham's F12 medium containing 10% fetal calf serum, 0.05% gentamycin and a mixture of 20 mM Hepes and 10 mM sodium bicarbonate buffers. Further details have been described previously (Raaphorst et al., 1985).

Hyperthermia was administered to cultured cells sealed in plastic tissue culture flasks and immersed in temperature controlled waterbaths ($\pm$0.02^{o}C). The heating time shown in the data indicate the total time at which the flasks were at the elevated temperature. The half-time for temperature equilibrium was about 30 seconds for cells sealed in T25 flasks.

Irradiation was done using a Siemens Stabilipan-2 x-ray machine operating at 250kV with a 1 mm Al filter and giving a dose rate of 3.0 Gy per minute.

All treatments were done with cells in exponential growth phase and unless otherwise indicated survival was assayed using standard colony forming assay and the error bars on the figures indicate the standard error of the mean of 4 to 8 replicate points. More details of the experimental technique can be found elsewhere (Raaphorst et al., 1986). The procedures for sample preparation for electron microscopy have been described in detail in previous reports (Szekely et al., 1982, 1983a,b, 1985).

Results and Discussion

Figure 1 shows the killing of Chinese hamster cells by hyperthermia at various temperatures. The results show that heat kills cells and that a 1.0^{o}C change in temperature in the critical range has a large effect on cell killing. At temperatures below 42.0^{o}C, survival curves show a plateau for prolonged heating times indicating a thermally resistant state referred to as thermotolerance and reviewed elsewhere (Henle & Dethlefsen, 1978) while at temperatures of 43.0^{o}C or higher, survival drops exponentially as a function of heating time. These results have been noted for many cell lines studied in culture (Sapareto et al.,

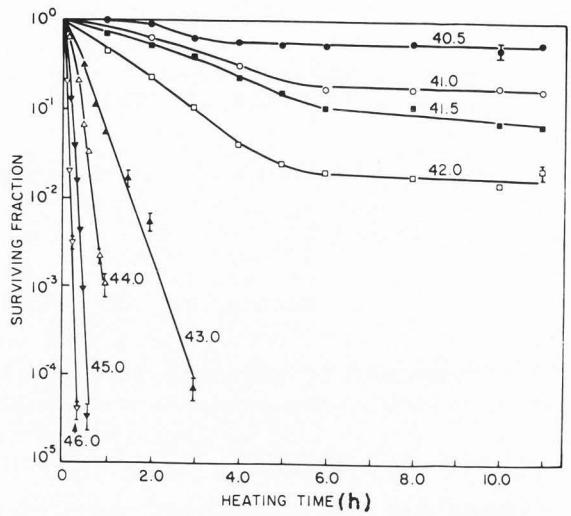


Figure 1. Survival of Chinese hamster V79 cells after heating.

1978a,b, Raaphorst & Azzam 1983a). It was shown in several other studies that thermal sensitivity could vary extensively among different cell lines. These differences were not correlated with DNA content, chromosome number, cell size or cell growth rate (Raaphorst et al., 1979b). Other studies have shown that differences in cellular thermal sensitivity may be related to membrane phospholipids and cholesterol (Cress et al., 1982) but thermotolerance was not related to these factors (Mendez et al., 1982). However our results with transformed mouse cells showed no correlation of the above membrane factors to thermal sensitivity (Raaphorst et al., 1985). There is some further evidence that hyperthermia damage may occur at the membrane level resulting in blebbing (Kapiszewska & Hopwood 1986, Borelli et al., 1986) and at the nuclear level resulting in changes in nucleolus, chromosome structure and protein content (Simard & Bernhard 1967). The study by Borelli et al (1986) showed a correlation between blebbing and cell death in cells with blebs larger than 50% of the cell for cells heated at 45.5^{o}C. It is of interest to compare the effects of hyperthermia (a radiosensitizer) to the effects of diamide or diethylmaleate which are radiosensitizers and also cause extensive blebbing of the cellular membrane (Szekely & Lobreau, 1987). Wachsberger and Coss (1987) showed a correlation between irreversible disruption of cytoskeletal networks and killing of CHO cells heated to 45.0^{o}C. These morphological effects should be further investigated using

microscopy and correlated with cell killing under a wide range of hyperthermia conditions and in combination with radiation.

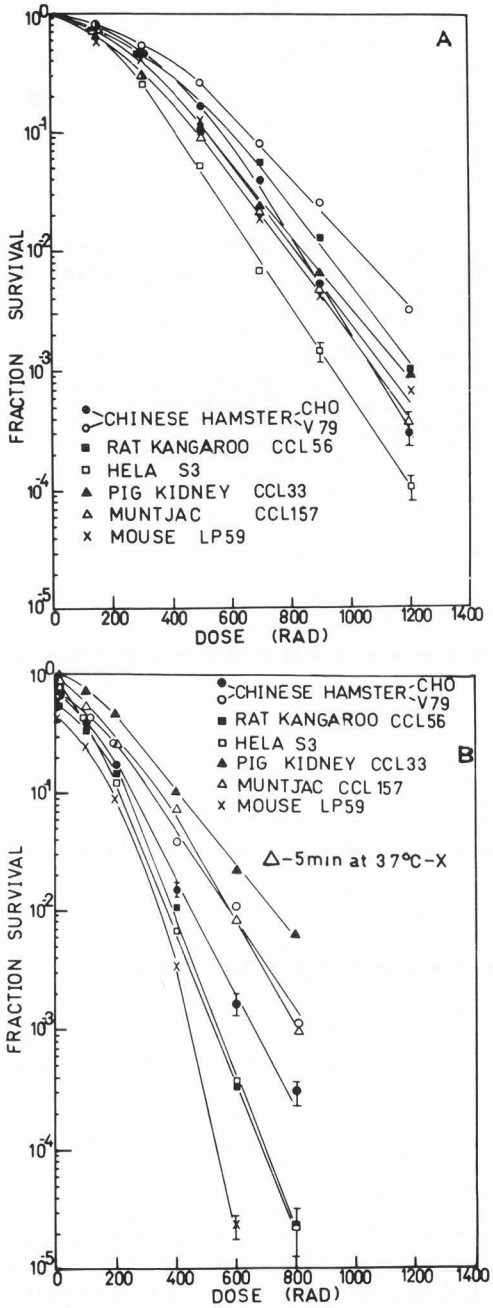


Figure 2a. Survival of various cell lines after irradiation (Raaphorst et al., 1979b with permission).

Figure 2b. Survival of various cell lines after heat (△ = 42.5°C for 1 h) and x-rays administered 5 minutes after heating. The cells were all cultured in Ham's F12 medium (Raaphorst et al., 1979b with permission).

Figures 2a,b show radiation sensitivity and radiosensitization in seven cultured cell lines. The range in radiation sensitivity (Figure 2a) is amplified by a small heat treatment (Figure 2b). Thus, hyperthermia may increase the range in radiosensitivity among various cell types and have important clinical implications when the treatment area contains different tissues and cell types. In earlier studies radiosensitization by hyperthermia was correlated to increased chromosomal aberrations in one cell line (Dewey et al., 1978). This work should be extended to include more cell lines of differing sensitivities and conditions of protection and sensitization.

Radiation resistant cells can pose a major problem in the use of radiation in cancer treatment and some conditions that prevail in tumors can cause increased radiation resistance. Several studies have shown that conditions that lead to radioresistance do not lead to thermal resistance but instead cause thermal sensitivity. Therefore, hyperthermia could play a complementary role to radiation in cancer therapy.

The state of hypoxia is known to make cells radioresistant and such radioresistance can lead to failure in cancer therapy for tumors containing hypoxic cells (Kallman 1972). Figure 3 shows the difference in radiation sensitivity of Chinese hamster cells irradiated under aerobic or hypoxic conditions. Figure 4 is an illustration of results redrawn from Gerweck et al., (1981) indicating that aerobic and hypoxic cells display no difference in thermal sensitivity. Table 1 summarizes the results of reports on the effect of hypoxia on heat sensitivity and thermal enhancement of radiosensitivity (Gerweck et al., 1981, Gerweck et al., 1974, Mivechi et al., 1981, Kim et al., 1983, Robinson et al., 1974, Durand 1978, Bass et al., 1978, Nielsen 1981, Rajaratnam et al., 1981, Power & Harris 1977, Overgaard & Bichel 1977, Gerweck et al., 1979). In the majority of these studies, hypoxic cells were equally heat sensitive or more sensitive than aerobic cells. However, two studies showed hypoxic cells to be more thermally resistant than aerobic cells. The study by Gerweck et al., (1979) showed that the OER was 1 for acute hypoxia but less than 1 for chronic hypoxia which may more closely model the clinical situation. However, many of the other studies presented in Table 1 also indicate an OER of less than 1 for acute hypoxia and thermal cell killing. Some studies also showed that for combined heat and x-ray treatments, hyperthermia caused a reduction in the oxygen

TABLE 1. EFFECT OF HYPOXIA COMBINED WITH HEAT AND RADIATION

Reference	Cells	Temperature °C	Hypoxia	OER Heat Alone	OER X Alone	OER Heat + X[1]
Gerweck et al., 1974	CHO	45.5	Acute	< 1	2.5	--
Mivechi et al., 1981	BP-8	37-45	Acute	1	Normal	Normal X△
Kim et al., 1983	HeLa	40.5	Acute	--	Normal	3.2 X△
Robinson et al., 1974	Bone Marrow	42.5 43	Acute	--	2.47	1.69 (X△) 1.38
Gerweck et al., 1981	CHO	43	Acute	1	3.1	
Durand, 1978 V79 Spheroids	Small Large	42-46	Acute	1 < 1	Normal	
Bass et al., 1978	HeLa CHO	42,43	Acute	> 1 1	3.1 3.1	
Nielsen, 1981	L1A2	42	Acute 0-24 h	1	Normal	
Rajaratnam et al., 1981	V79	43 44	Acute 16 h	> 1 > 1		
Power and Harris, 1977	V79 EMT6	43	Acute	1 1	3.0 3.2	3.8 △X 2.9 △X
Overgaard and Bichel, 1977	JB-1-E	42.5	Acute	< 1		
Gerweck et al., 1979	CHO	42	Acute Chronic	1 < 1		

[1] △ = heat treatment, X = X-rays

enhancement ratio (OER), while most results indicated that the OER remained unchanged. Variation in the oxygen effect may be related to variation in nutrient level of the culture medium. Results presented in a study by Kim et al., (1978) show that, while the OER for x-rays was not affected by the glucose content in the medium, the OER for thermal cell killing was strongly influenced by the presence or absence of glucose in the medium. That is, cells became more sensitive to hyperthermia under conditions combining hypoxia and absence of glucose. These results and others (Gerweck et al., 1984) indicate that hypoxia and nutrient factors together may play an important role in cellular thermal sensitivity.

Cells become thermally sensitive in the absence of culture medium as shown in Figure 5. A large increase in Chinese hamster cell killing occurs from heating in buffered (pH 7.4) isotonic salt solutions (Raaphorst et al., 1984, Raaphorst & Dewey 1978). The presence of glucose did not diminish cellular heat sensitivity. Thus, the absence of certain requirements supplied in culture medium resulted in potentiation of thermal sensitivity. Others have also shown the importance of cellular nutrients in thermal sensitivity of cells (Hahn 1974, Li et al., 1980). This effect may have a relationship to osmotic factors due to enhanced membrane damage. The data of Coss et al., (1979) showed that heating may cause an osmotic imbalance in the cell resulting in swelling in mitochondria. Our own results also indicate changes in cell size during heating (Raaphorst & Dewey, 1978). Further investigation is needed to evaluate cell and organelle size and structure under various conditions of heating.

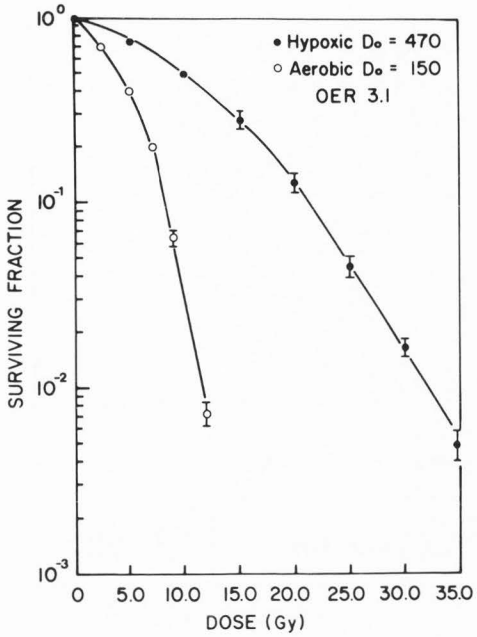


Figure 3. Survival of V79 cells irradiated under aerobic or hypoxic conditions. OER = oxygen enhancement ratio = D_o hypoxic/D_o aerobic.

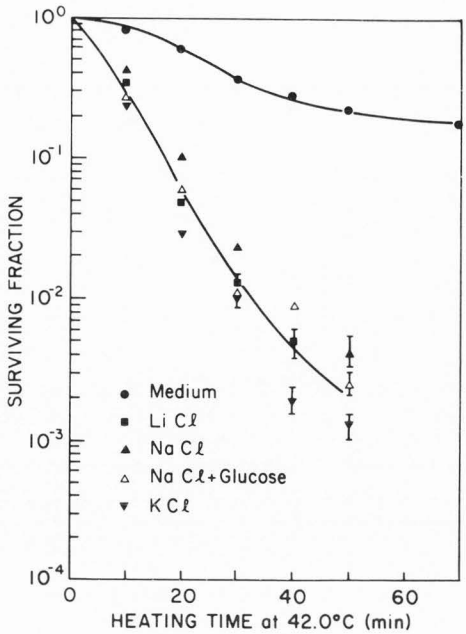


Figure 5. The effect of exposure of CHO cells to monovalent salt solutions at 42.0°C for the conditions, as indicated in the Figure.

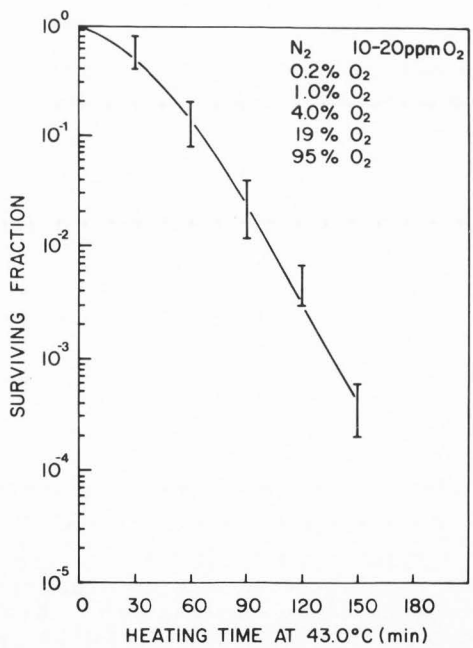


Figure 4. Survival of CHO cells heated under hypoxic or aerobic conditions. Data randomly fluctuated within the error bars. Data redrawn from Gerweck et al., (1981).

When cells are held in a nutrient-deprived state after x-irradiation (Figure 6), repair of radiation damage occurs causing an increase in radiation resistance (Raaphorst & Azzam 1981, Hetzel et al., 1976). Thus, cells which become radioresistant through repair of damage during nutrient depletion are also more heat sensitive.

The pH of the cellular environment also plays a role in x-ray and thermal sensitivity. Figure 7 shows that Chinese hamster cells are more resistant to radiation at low pH (Freeman et al., 1981). This effect was also observed in human glial (Astrocytoma) cells (Rottinger et al., 1980). For thermal sensitivity the opposite is true; cells at acidic pH are more thermally sensitive than cells at normal pH of 7.4 as shown in Figure 8 for Chinese hamster cells (Freeman et al., 1980, Gerweck & Rottinger 1976). Since tumor pH was shown to be lower than normal tissue pH (Meyer et al., 1948, Kahler & Robertson 1943, Gullino et al., 1965), the thermal sensitization by an acidic pH can be a major factor in enhanced cell killing in thermal cancer therapy of tumors having low pH levels. The thermal sensitization of cells at low pH has been shown in many experiments (Gerweck

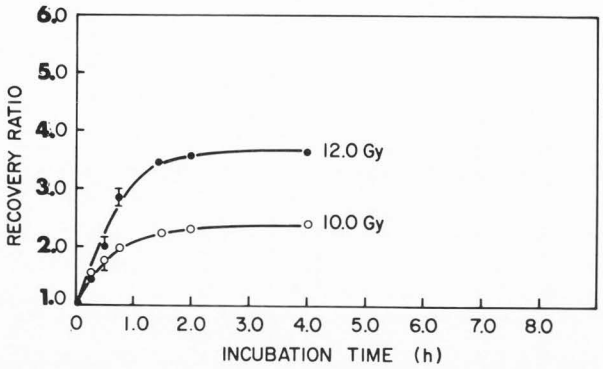


Figure 6. Repair of potentially lethal radiation damage for V79 cells held in Earle's balanced salt solutions at 37°C after irradiation as indicated on the abscissa.

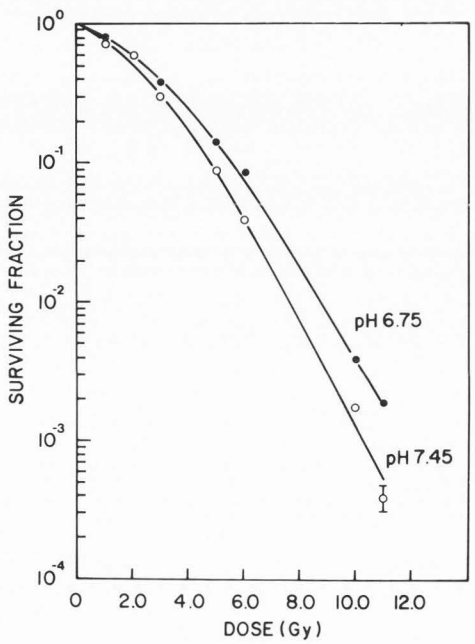


Figure 7. The effect of pH on radiosensitivity of CHO cells. The pH was reduced 1 h before irradiation by increasing the CO_2 concentration. Data redrawn from Freeman et al., (1981).

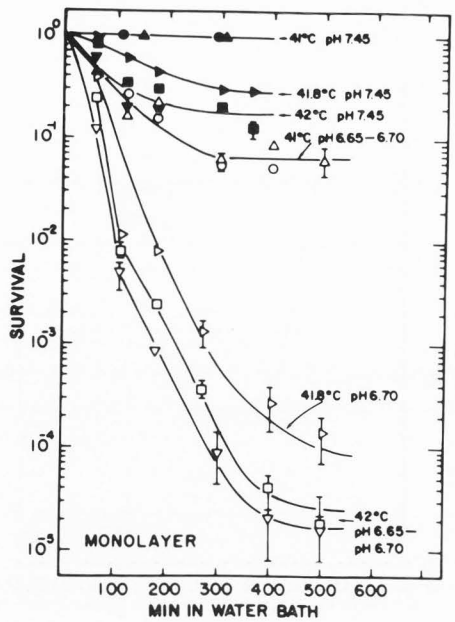


Figure 8. Survival of CHO cells heated in medium at alkaline or acidic pH. The pH was changed 2 h before heating (Freeman et al., 1981 with permission).

& Rottinger 1976, Hofer & Mivechi 1980, Bichel & Overgaard 1977), and there is evidence that the induction of hyperglycemia may further reduce tumor pH and thus increase thermal sensitivity (Urano et al., 1983). The thermal enhancement of radiosensitivity can also be further increased at low pH as shown in our earlier studies (Figure 9 results redrawn from Freeman et al., 1981). These data show that the decrease in the slope (D_o) of the radiation survival curve was much greater for heating for 1 h at 42.0°C at pH 6.63 than at pH 7.43. Note also that the heat treatment alone caused a greater degree of cell killing at pH 6.63 compared to pH 7.43, thus it cannot be determined whether increased thermal enhancement of radiosensitivity was due specifically to the acidic pH or more generally due to an increase in thermal cell damage. Similar results were also observed in murine mammary carcinoma cells (Haveman 1983b).

Variation of cellular sensitivity to x-rays and heat also occurs for cells in various stages of the cell cycle (Raaphorst & Azzam 1984a, Westra & Dewey 1971, Bhuyan et al., 1977). Figure 10 shows that Chinese hamster cells are more resistant to x-rays in S-phase than in G_1 phase and that this difference in x-ray sensitivity can be reduced by giving a heat treatment before irradiation. Similar results were obtained irrespective of whether cells were synchronized by mitotic selection, a double excess thymidine block or by hydroxyurea (Raaphorst & Azzam 1984a). Figure 11 further shows that at the stages of the cell cycle in which cells are radiation resistant the thermal resistance is low and when thermal

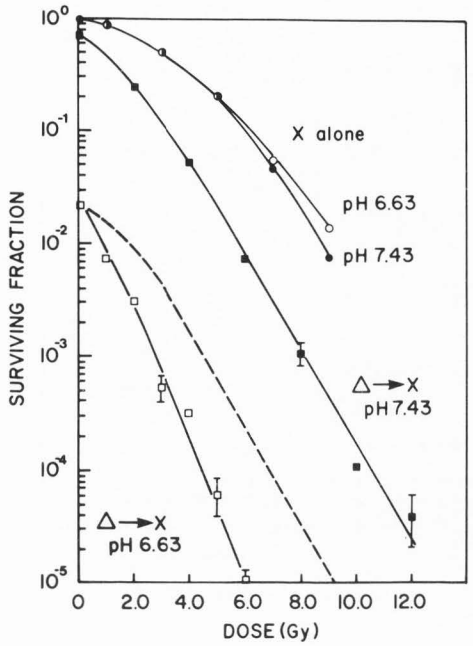


Figure 9. The survival of CHO cells after heating or heating plus irradiation in alkaline or acidic medium. The dashed curve is the X, pH 7.43 curve brought down to the same level as the low pH, X curve for comparison. Heat (indicated by △) was 60 minutes at 42.5°C and irradiation occurred 2.5 h after heating. The pH was lowered 2 h before heating (Freeman et al., 1981 with permission).

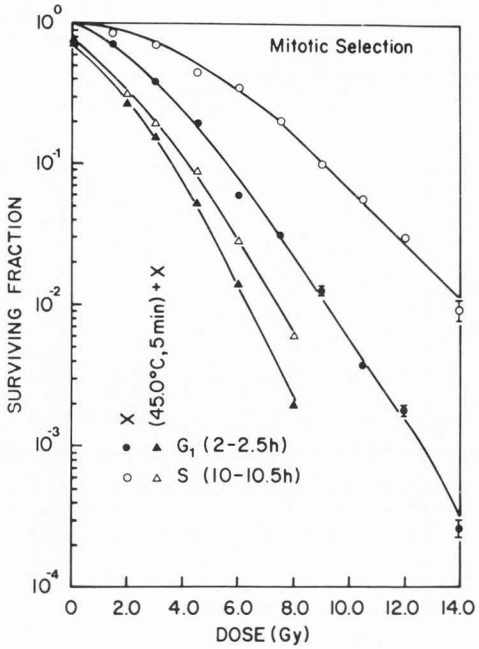


Figure 10. Survival of synchronous G_1 or S-phase cells after irradiation or heating (45.0°C, 5 minutes) plus irradiation. Heating was completed 10 minutes before irradiation. Cells were synchronized by mitotic selection (Raaphorst & Azzam, 1984a with permission).

resistance is high, radiation resistance is low. These data show the complementary nature of the heat and radiation responses. When heat and radiation are combined, the cell cycle variation is reduced and there is a synergistic interaction of heat and radiation damage which is greatest in S-phase cells (Figure 10). The cells were more sensitive to heat and radiation in mitosis which may imply mitotic death mechanisms. These were investigated by several researchers. The results show that hyperthermia caused disassembly of microtubules, desynchronization or arrest of chromosome movement, damage to centrosomes, formation of micronuclei, aberrant cell division and formation of tetraploidy (Coss et al., 1982, Barrau et al., 1978, Rieder & Bajer 1977, Rofstad et al., 1984, Zielke-Jemme & Hopwood 1982). Our own results (Szekely & Raaphorst, work in progress) indicate that colcemid can protect mitotic cells during heating. Further studies are needed to evaluate these effects in mitosis for heating at various times during the cell cycle and during thermosensitization, thermoprotection and the development of thermotolerance. In addition, a comparative study on heat and radiation damage is needed.

The conditions of hypoxia, low pH, nutrient depletion and S-phase in the cell cycle increase radiation resistance but do not affect thermal sensitivity (hypoxia alone) or increase thermal sensitivity. These conditions can be found in tumors and thus the preceding results provide a rationale for using hyperthermia to complement radiation in cancer therapy. Furthermore, hyperthermia and radiation damage interact to give a synergistic effect in cell killing which can be used to enhance the killing of tumor cells. Further studies should evaluate the differences of heat and radiation damage at the molecular and ultrastructural levels especially under conditions that modify sensitivity to heat and radiation in opposite directions.

In Figures 2a and b, the thermal enhancement of radiation sensitivity for radiation at high dose rates was shown. This effect can be enhanced when radiation is given at low dose rates as shown for hamster cells in Table 2,

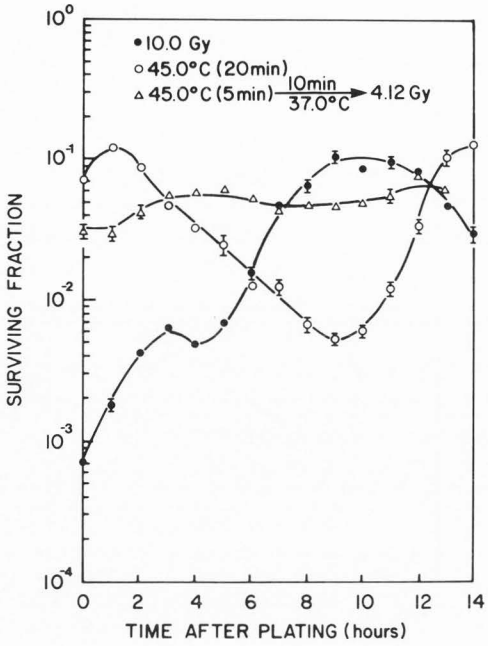


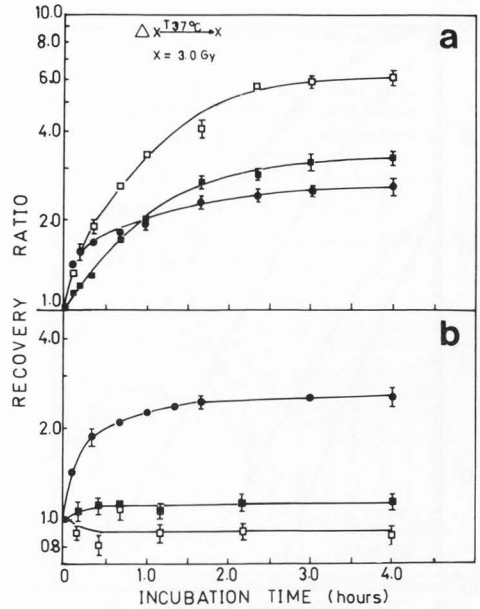
Figure 11. Survival of V79 cells heated and/or irradiated at various stages of the cell cycle. Cells were synchronized by mitotic selection (Raaphrost & Azzam, 1984a with permission).

TABLE 2. HYPERTHERMIA AND RADIATION: DOSE RATE EFFECT

Reference	Dose Rate rad/min	Temperature °C	Do 37°C / Do T°C
Ben Hur	360	37	1.0
et al.,	360	42	1.5
1974	12	37	1.0
	12	42	3.3
	3.3	37	1.0
	3.3	42	6.3
Harisiadis	94	37	1.0
et al.,	94	41	1.6
1978	3.3	37	1.0
	3.3	41	1.9

Irradiation was done during heating for combined treatments.

based on data taken from two research reports (Harisiadis et al., 1978, Ben Hur et al., 1974). These data show that at low dose rates the reduction in the radiation survival curve slope (D_o) is much larger than at high dose rates. Thus, hyperthermia would provide a



Figures 12a&b. The effect of heat (completed 10 minutes before irradiation to 3.0 Gy) on repair of sublethal radiation damage in V79 cells assayed by incubation between two x-ray doses. (a) represents the small heat treatments causing only 10% cell killing (open squares, 42.5°C, 30 min; closed squares, 45.5°C, 4 min; closed circles, no heat). (b) represents the results for severe heat treatments reducing survival by 90%. Heating terminated 10 minutes before irradiation (open squares, 42.5°C, 120 min; closed squares 45.5°C, 15 min; closed circles, no heat).

greater effect for low dose rate radiation which occurs during radiotherapy administered by radioactive implants. The low dose rate data imply that the heat treatment may block a radiation damage repair system that repairs sublethal radiation damage during low dose rate exposure. The results in Figure 12 show that hyperthermia does not inhibit repair of sublethal radiation damage (SLD) when small heat treatments (survival reduced by 10-20%) are given before irradiation while larger heat treatments given before irradiation (survival reduced by 90%) block the repair of SLD. These results agree with data published earlier for Chinese hamster ovary cells (Raaphorst et al., 1979a). Radiation resistance can vary extensively among cell lines derived from different tumor or normal tissues. This is clearly illustrated in Figure 13 which shows a

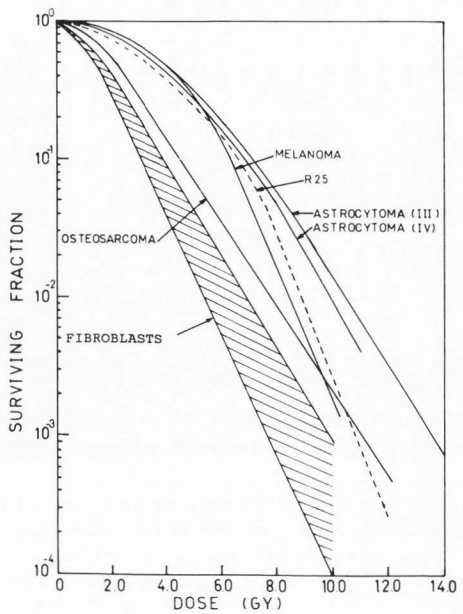


Figure 13. Radiation survival of human normal fibroblasts and human tumor cells. The area between the cross-hatched lines indicate the range in sensitivity of the fibroblasts. R25 represents a melanoma cell line transformed from a mouse parental cell line in culture (Redrawn from Gerweck et al., 1977).

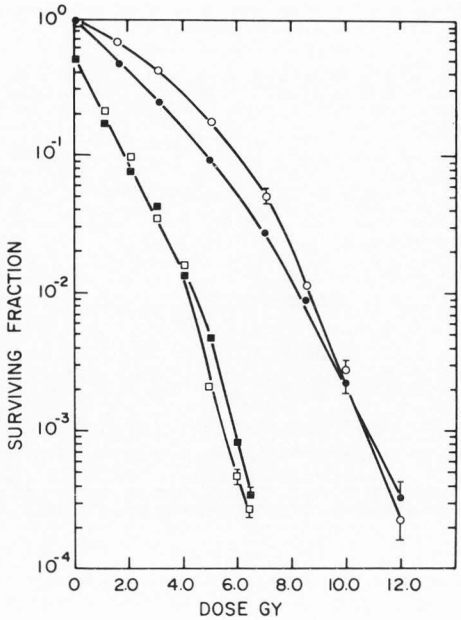


Figure 14. Survival of normal mouse C3H-10T1/2 cells and R25 transformed cells after irradiation (closed circles and open circles, respectively) or heat plus radiation (closed squares and open squares, respectively). Combined treatment was 10 min at 45.0°C which was completed 10 min before irradiation. The thermal enhancement on radiosensitivity was greater at the low dose region (up to 4.00 Gy) for R25 (Raaphorst et al., 1986 with permission).

summary of our own data and results taken from the literature (Raaphorst et al., 1986, Raaphorst & Azzam 1985, Gerweck et al., 1977). The large shoulders on the survival curves indicate a large capacity for accumulation and possibly repair of sublethal radiation damage which can lead to radioresistance in fractionated cancer radiotherapy (Weichselbaum et al., 1982, Barranco et al., 1971). Hyperthermia can overcome this resistance as shown in Figure 14 for R25 cells. R25 cells were developed in our laboratory and display many characteristics of malignant melanoma cells including radioresistance (Raaphorst et al., 1986, Szekely et al., 1985). The R25 cells, seen in Figure 15 show a transformed phenotype with a large number of cells growing on top of each other. This lack of contact inhibition is contrasted by the smooth, confluent growth seen in the normal C3H-10T1/2 cells (the parental strain) (Figure 16). Heat treatment caused synergistic enhancement of radiosensitivity and resulted in reducing the survival curve shoulder to the same level as the normal parental mouse cell line. These results indicate that hyperthermia may have the ability to reduce or eliminate differences in radiation responses in cells and tissues stemming from differences in repair capacity. At the present time, the mechanism of radioresistance in these cells remains unknown. Our results indicate R25 cells contain melanosomes (Figure 17) and melanin but these do not appear to be associated with resistance (Szekely et al., 1985, Raaphorst et al., 1986). Other studies show that hyperthermia treatment can inhibit the activity of B polymerase which is active in repair of radiation damage (Jorritsma et al., 1986, Mivechi & Dewey 1985). More studies are needed on the molecular and morphological level to determine the underlying mechanisms of altered sensitivity or radiosensitization. Such results could lead to models to be used in predicting sensitivity and providing means to improve sensitivity to treatment in human cancer.

Repair of potentially lethal

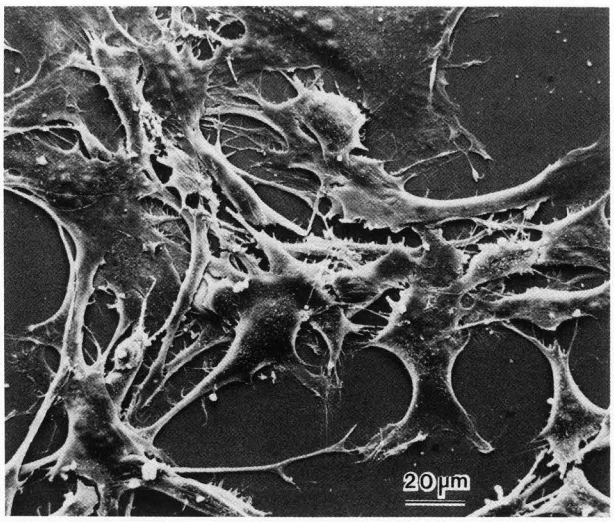


Figure 15. A scanning electron micrograph of R25 cells. They were developed from a transformed focus of C3H-10T1/2 cells. Cells are growing over each other.

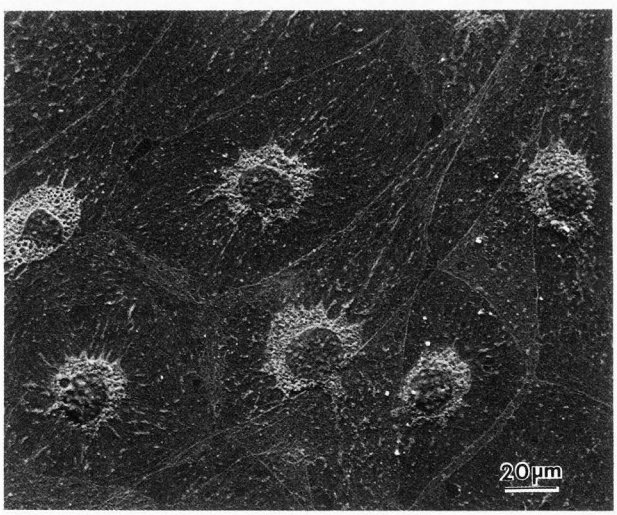


Figure 16. A scanning electron micrograph of normal, untransformed C3H-10T1/2 cells. Note that the cells grow in a characteristic monolayer.

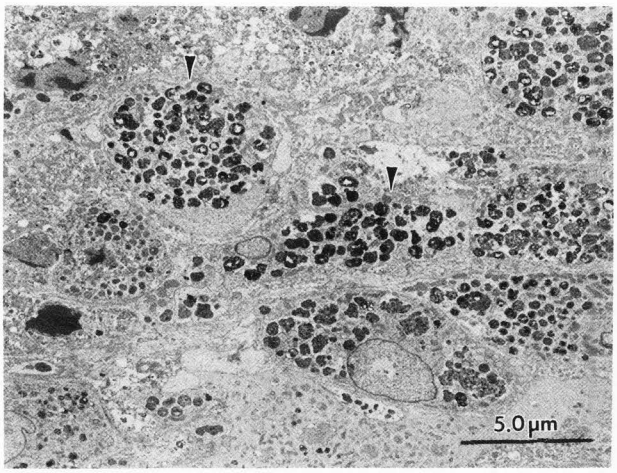


Figure 17. A transmission electron micrograph of an area near the surface of a dark spheroid of R25 cells. The pigment-filled melanosomes are visible (arrows). Szekely et al., 1985 with permission.

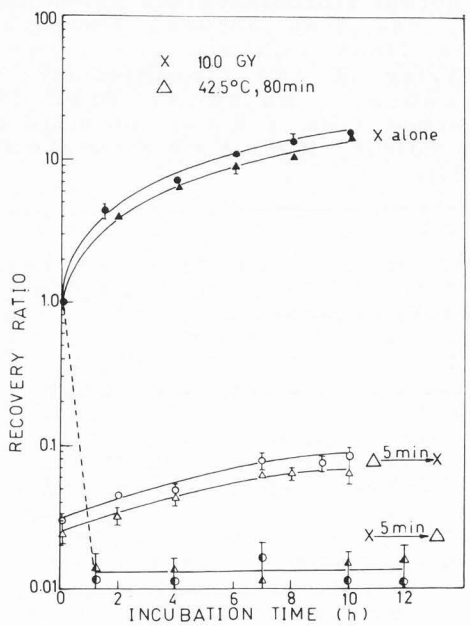


Figure 18. Repair of PLD of normal (circles) and transformed (triangles) C3H-10T1/2 cells held in plateau phase after treatment for various times before trypsinization and plating. Heat was given 5 min after irradiation or terminated 5 min before irradiation. Recovery ratio is the ratio of survival at time (incubation) after treatments divided by the survival for cells plated immediately after irradiation alone.

radiation damage (PLD) occurs in cells and tissues. This recovery of PLD in vitro and in vivo can result in large increases in survival (Little et al., 1973) and reduce the effectiveness of radiation in cancer treatment. The blocking of radiation repair systems could thus enhance the effectiveness of radiation in cell killing during radiation cancer therapy. The results in Figure 18 show that hyperthermia can inhibit repair of PLD. The data show

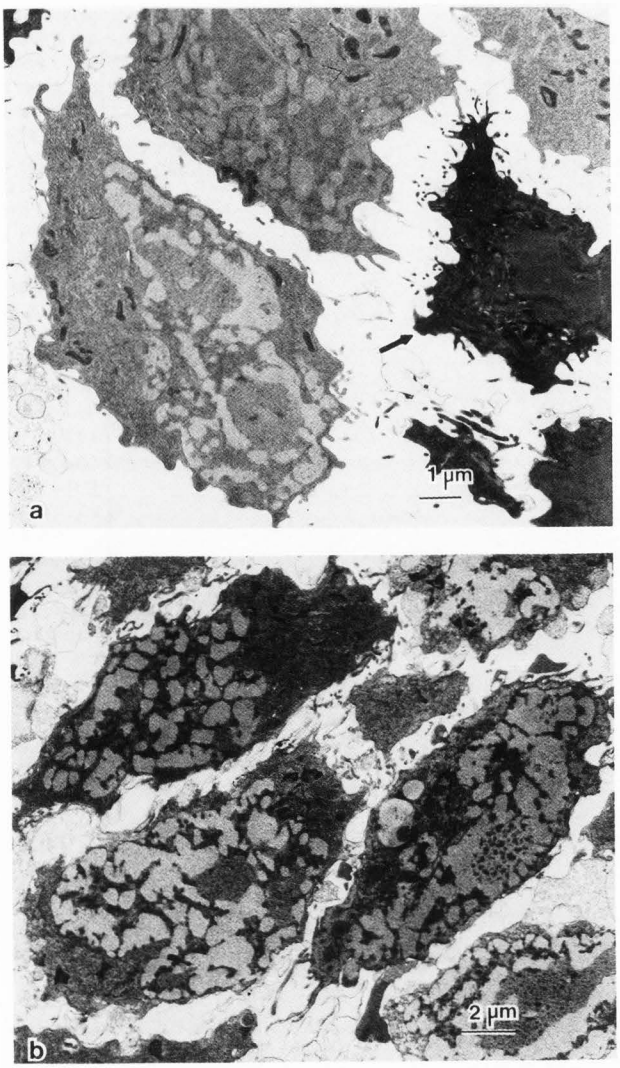


Figure 19. Transmission electron micrographs of a V79 Chinese hamster cells exposed to 1.5 M NaCl for: (a) 10 min at 22°C or (b) 30 min at 37°C. Darkly staining cells with many microvilli-like projections, such as the cell marked by the arrow in (a) are seen in cells treated at 22°C. These cells are less radiation-sensitive than similar cells treated with 1.5 M NaCl at 37°C.

that hyperthermia increased the level of cell killing and also reduced the subsequent ability of cells to repair PLD during incubation in plateau phase after combined treatments. Repair inhibition was greater for heating after irradiation compared to heating before irradiation and this inhibition was greater for heating at low temperatures for longer heating times (Raaphorst et al., 1987a). Earlier studies by Li et al., (1976) also show a similar effect

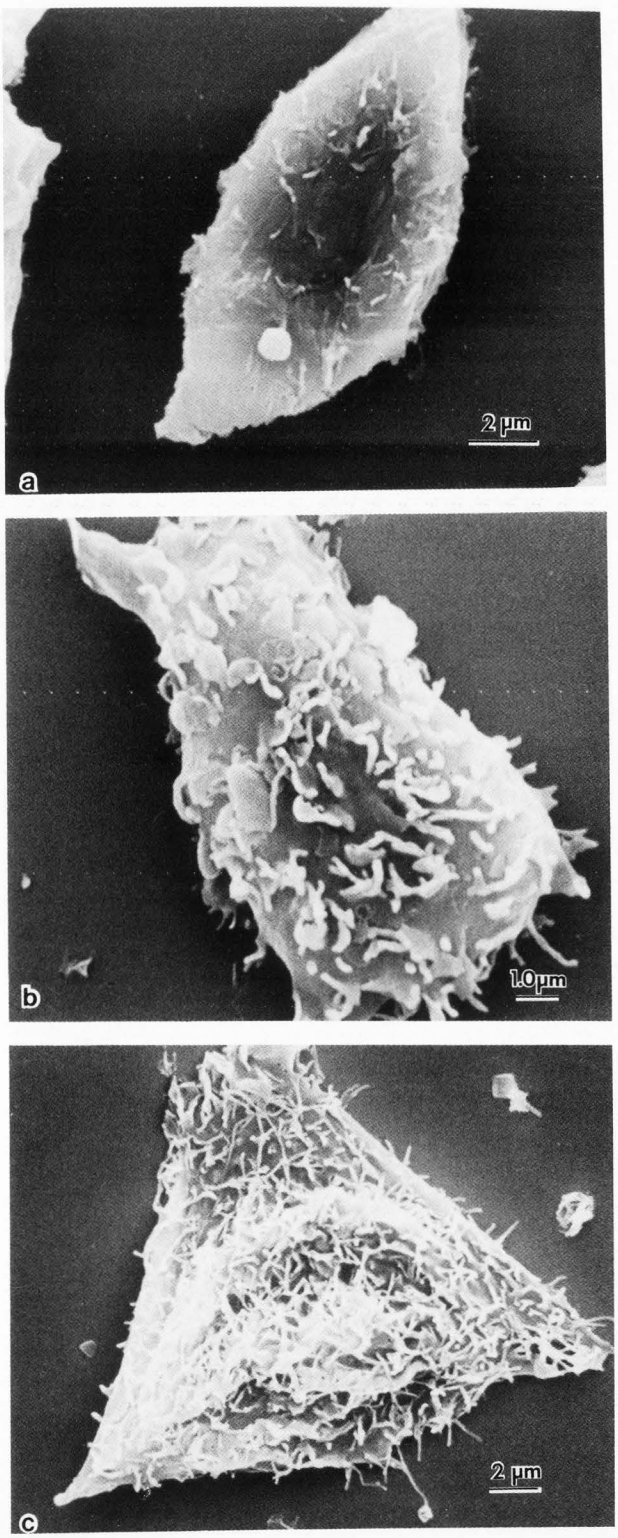


Figure 20. Scanning electron micrographs of V79 Chinese hamster cells treated with anisotonic NaCl; (a) control cell sham-treated with medium, (b) a 0.5 M NaCl exposure for 20 min, (c) a 1.5 M NaCl exposure for 20 min.

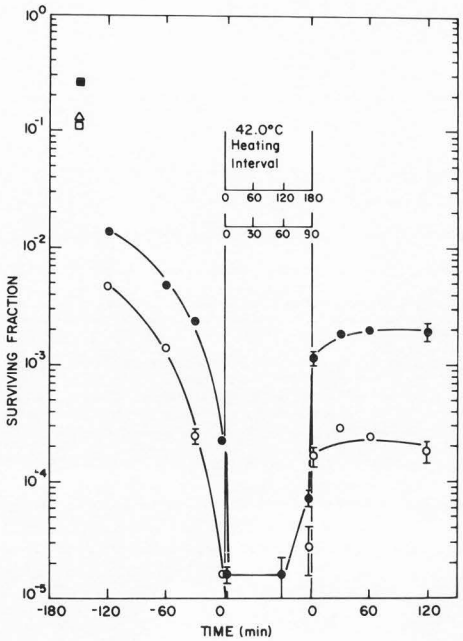


Figure 21. The survival of V79 cells after treatment with sequences of x-rays before heating (negative abscissa); x-rays during heating, (between vertical bars) and x-rays after heating (positive abscissa). Cells were held at 37°C between treatment. The symbols are as follows: closed circles, 1.5h at 42.0°C plus 6.0 Gy; open circles, 3.0 h at 42.0°C plus 6.0 Gy; closed square, 1.5 h at 42.0°C; open square, 3.0 h at 42.0°C; open triangle, 6.0 Gy.

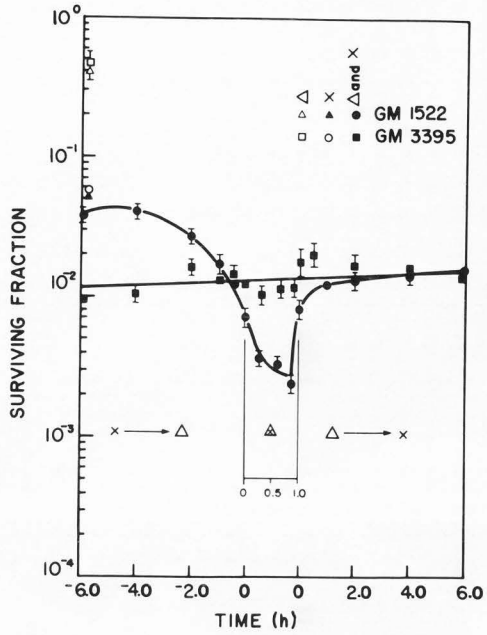


Figure 22. Sequencing of heat and radiation treatments for human normal (GM1522) or ataxia telangiectasia (GM3395) cells. Presentation is the same as described in Figure 16 and heating was 1 h at 42.0°C and x-ray was 5.0 Gy for GM1522 and 1.5 Gy for GM3395, respectively (Raaphorst & Azzam, 1984b with permission).

in Chinese hamster ovary cells. Thus one mechanism of thermal radiosensitization is through the inhibition of repair of sublethal and potentially lethal radiation damage. Our data using anisotonic treatments show that repair after irradiation can be inhibited and is accompanied by large changes in chromatin structure (Figure 19) implying that such conformational changes may be responsible for repair inhibition (Raaphorst & Dewey 1979, Szekely et al., 1982, 1983a,b). The anisotonic treatment is also reflected in the surface structure of the Chinese hamster cells, Figure 20. The shrinkage of the cell, with perhaps some post-treatment reswelling, is shown by the increase in microvilli-like projections on the cell surface. Some studies have already shown that hyperthermia can also affect nuclear, nucleolus and chromatin structure (Coss et al., 1982, Barrau et al., 1978, Rieder & Bajer 1977, Clark et al., 1981a).

Further studies are needed to correlate such changes with inhibition of capacity to repair radiation damage.

The thermal enhancement of radiation sensitivity for low and high dose rate radiation (Raaphorst et al., 1979a,b, Harisiadis et al., 1978, Ben Hur et al., 1974, Dewey et al., 1980) depends on the sequence of the radiation and heating (Sapareto et al., 1978a, Raaphorst & Azzam 1983b, 1984b). The data in Figure 21 show that in Chinese hamster cells, maximum thermal enhancement occurred during the simultaneous application of heat and x-rays. When x-rays were given before heating, survival increased as incubation time at 37°C was prolonged between the two treatments. This was also observed for heating followed by x-rays and these data demonstrate that cells can recover from heat damage which interacts with x-ray damage or vice versa.

The sensitivity of cell killing to the sequence of heat and radiation is even greater for prolonged heating at lower temperature hyperthermia (Raaphorst et al., 1987a). Heating before irradiation resulted in thermal

enhancement of radiation sensitivity for temperatures of 41.5°C or higher while heating after irradiation resulted in increased radiation sensitivity for temperature as low as 40.5°C. This effect may be due to the conversion of potentially lethal damage to lethal damage by heating after irradiation which cannot occur during heating before irradiation. While this sequencing effect is also observed in normal human cells (Figure 22) it is absent in the radiation sensitive ataxia telangiectasia (AT) cell strain (Raaphorst & Azzam, 1984b). AT cells are known to have deficiencies in radiation damage repair systems (Paterson & Smith, 1979) and these may be involved in the lack of recovery during incubation at 37°C between the two treatments of heat and x-rays or vice versa. This lack of sequence dependence has also been observed in a human T leukemia cell line (Raaphorst et al., 1983). Further studies into the response of B polymerase and evaluation and comparison of nuclear morphological response to hyperthermia in these recovery deficient cell lines may provide further insight into the nature of thermal damage and radiosensitization.

Recovery during incubation at 37°C between heat and x-ray treatments can be inhibited by low pH or low oxygen levels (Freeman et al., 1981, Ducoff 1982) conditions that can exist in tumors. Figure 23 (data redrawn from Freeman et al., 1981) shows that by decreasing the pH from 7.4 to pH 6.7, recovery between a 1 h heat treatment at 42.5°C and a 5.0 Gy, x-ray dose is reduced from a factor of 6 to 1.5. Thus, cells and tissues at normal pH 7.4 would recover between treatments while tumor cells at low pH would not recover and be preferentially killed. This would provide a therapeutic gain for treatment of tumor versus normal tissue.

Thermal enhancement of radiation sensitivity also depends on the temperature of the heat treatment (Sapareto et al., 1979) and on the severity of the heat treatment (Raaphorst et al., 1979a, Raaphorst & Azzam 1983b, Joshi et al., 1978, Holahan et al., 1984). Heating Chinese hamster V79 cells at 42.0°C before irradiation (Figure 24) resulted in a continuous increase in radiation sensitivity even when cells become thermotolerant (Raaphorst & Azzam 1983b, Raaphorst et al., 1980). However, in CHO cells thermosensitization ceased to increase further when cells reached thermotolerance (Freeman et al., 1979). These data indicate that thermotolerance development during prolonged heating may produce varying degrees of

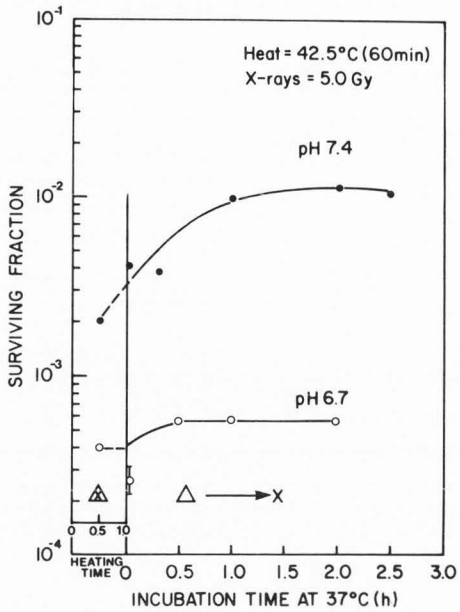


Figure 23. Recovery of CHO cells during incubation at 37°C, at pH 7.4 or 6.7 between heat and x-ray treatments. The pH levels were maintained during the heating, incubation and irradiation treatment time (Freeman et al., 1981 with permission).

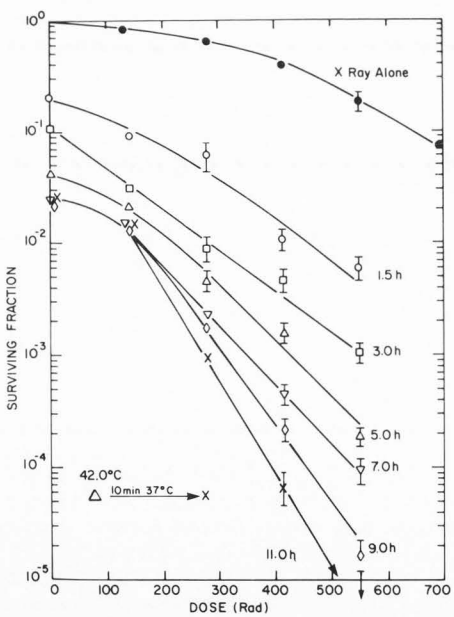


Figure 24. Survival of V79 cells after heating from 0-11 h at 42.0°C followed by x-rays 10 min after the termination of heating.

TABLE 3. THERMAL SENSITIVITY OF NORMAL AND MALIGNANT CELLS

Reference	Cell Types	Temperature °C	Relative[1] Sensitivity
Giovanella et al., 1976	Melanocytes: Melanoma Intestinal: Colon Carcinoma Fibroblasts: Fibrosarcoma Neuroepithelial: Terotocarcinoma human cells	43 43 43 42.5	T > N T > N T > N T > N
Giovanella et al., 1973	Mouse embryonal: MCA-induced sarcomas	42.5	T > N
Hahn, 1980	C3H-10T1/2 Normal: Transformed *Not correlated with the degree of malignancy	41-43.5	T > N*
Symonds et al., 1981	Mouse Haemopoietic Stem Cells: Leukemic L1210	43	T ~ N
Okumura et al., 1979	Human Normal: Malignant	39-44	T > N
Chen and Heidelberger, 1969	Mouse Prostate Normal: Transformed	43	T > N
Levine and Robbins, 1970	Human Normal: Cancer	42	T > N* *Plateau Phase
Kase and Hahn, 1976	Human Normal: SV 40T Transformed	43	T > N log T ~ N plateau
Auersperg, 1966	Human Fibroblasts: Carcinoma (epithelial)	46	T < N
Kachani and Sabin, 1969	Hamster Normal: X-ray Transformed Normal: Drug Transformed Normal: Virus Transformed	41.6	T ~ N T ~ N T > N
Robbins et al., 1983	AKR Mouse Bone Marrow: Leukemia	41.8 42.5	T > N
Raaphorst et al., 1985, 1987b	C3H-10T1/2 Normal: X-ray Transformed Normal: HRas Transformed	42-45	T < N, T = N, T > M, T = N

[1] T = tumor or Transformed cell; N = Normal cell

radiosensitization for different cell lines during this period. The development of thermotolerance during incubation between two heat treatments can also result in a loss of radiosensitization (Raaphorst & Azzam 1983b, Haveman 1983a, van Rijn et al., 1984). The data depicted in Figure 25 show that 20 minutes of heating at 45.0°C reduced the radiation survival curve D_o from 1.43 Gy to 0.52 Gy while the D_o increased from 0.52 to 1.30 Gy for a 9 h incubation time between two 10 minute heat treatments. These data suggest that the therapeutic effectiveness of hyperthermia in radiosensitization could be rapidly lost during the onset of thermotolerance. Such effects have also been observed in vivo (Law et al., 1979, Marigold & Hume 1982).

Many environmental factors that may prevail in a tumor affect the thermal sensitivity of cells and such factors may obscure the actual sensitivity of cells. The data in Table 3 (Giovanella et al., 1973, 1976, Hahn 1980, Symonds et al., 1981, Okumura et al., 1979, Chen & Heidelberger 1969, Levine & Robbins 1970, Kase & Hahn 1976, Auersperg 1966, Kachani & Sabin 1969, Robbins et al., 1983) summarize some of the in vitro

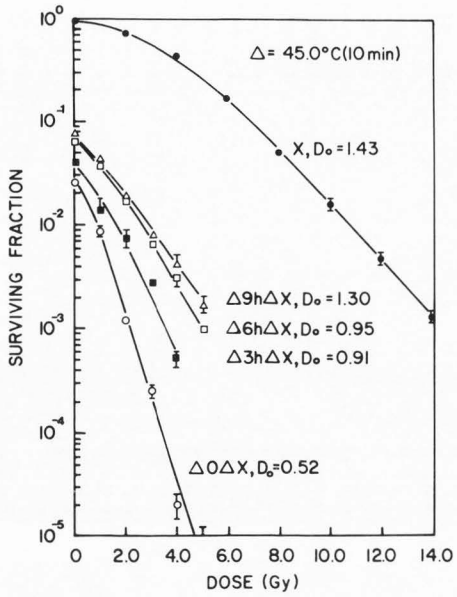


Figure 25. Survival of V79 cells after single or split heating at 45.0°C before irradiation. Irradiation followed 10 min after the last heat treatment. Incubation was at 37°C between heat treatments. The D_o for each survival curve is given in Gy (Raaphorst & Azzam 1983b with permission).

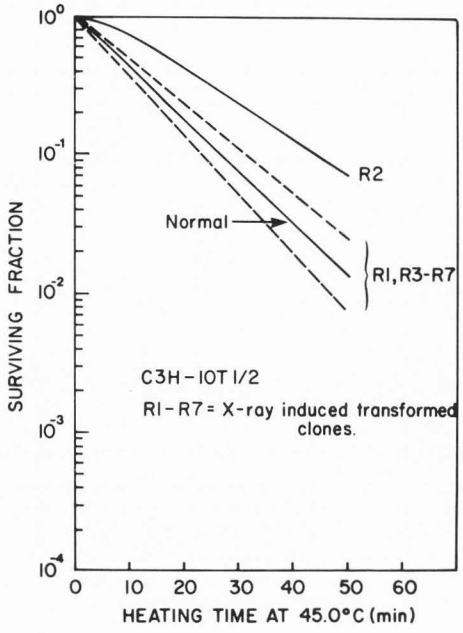


Figure 26. Survival after heating of C3H-10T1/2 cells; normal (the lower solid line) or transformed by x-rays (the upper solid line or falling between the dashed lines). Transformed cells formed spheroids in agarose and produced tumors in C3H mice while normal cells did not (Raaphorst et al., 1985 with permission).

studies comparing the thermal sensitivities of normal and malignant cells. Many of the studies indicate that transformed cells are more thermally sensitive than normal cells although some studies indicate no difference. Some of the documented differences may be related to the enhanced anaerobic glycolysis observed in transformed cells, which results in lactic acid production and acidification of the culture medium, thereby causing thermal sensitization. Our studies with the C3H-10T1/2 cell system show that cells transformed by x-rays (Figure 26) or by incorporation of oncogenes (Figure 27) display a range of thermal sensitivities, some of which are greater than the normal parental cell line and some which are less heat sensitive than the normal cell line (Raaphorst et al., 1985, 1987b). Figure 28 shows the range of transformed phenotypes seen in selected transformed cell lines derived from C3H-10T1/2 cells. These are to be compared with the C3H-10T1/2 line shown in Figure 16. The heating survival curves of these clones are summarized in Figure 26. Thus, the question of differences in thermal sensitivity of normal and transformed cells is not yet resolved. Such differences, where they exist, may depend on the nature of the normal cell and its transformed counterpart. Studies on the effect of heat on thermal enhancement of radiosensitivity of normal versus transformed cells are also needed to extend the knowledge in this area to guide the clinical use of hyperthermia. Several studies have shown that thermal damage is expressed on a morphological and ultrastructural level (Kapiszewska & Hopwood 1986, Simard & Bernhard 1967, Coss et al., 1982, Rieder & Bajer 1977, Wachsberger & Coss 1987, Borreli et al., 1986). Little or no work has been done to compare these endpoints in cells of different thermal sensitivity. Such a study would provide correlation between such changes and thermal sensitivity leading to elucidation of mechanisms.

Most of the methods of cancer treatment such as irradiation and chemotherapy can transform cells from a normal to a malignant state (Heidelberger et al., 1983, Borek & Hall 1973). We have tested hyperthermia in the mouse C3H-10T1/2 cell system to determine if heat or heat plus radiation transforms cells from the normal to the

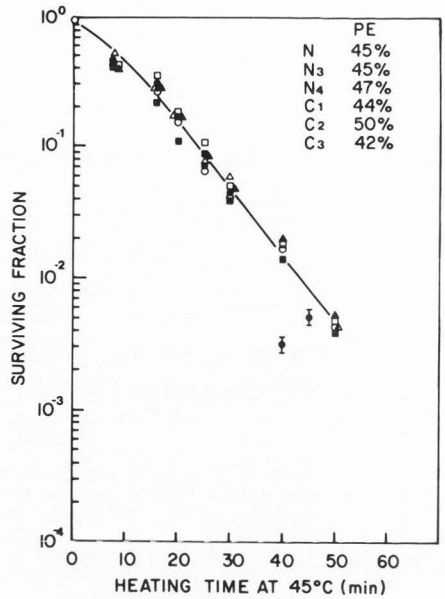


Figure 27. Survival of C3H-10T1/2 cells after heating; N = normal cells; N3 and N4 = cells transfected with HRas oncogenes but expressing a normal phenotype, C1-C3 = cells transfected with HRas oncogenes, overtly expressing a transformed phenotype and highly malignant, producing tumors in syngeneic mice (Raaphorst et al., 1987b with permission).

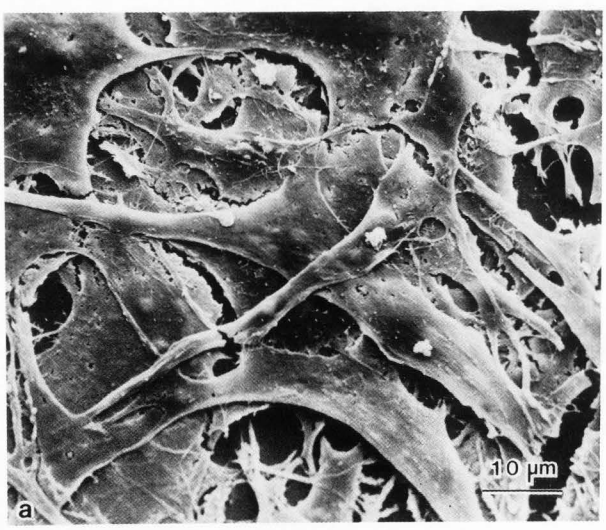
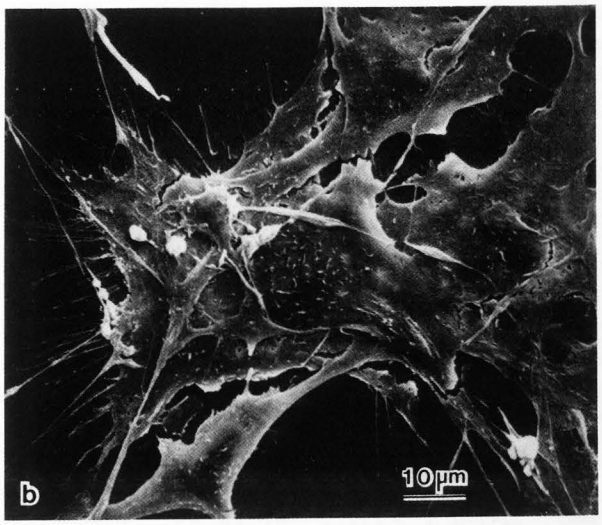
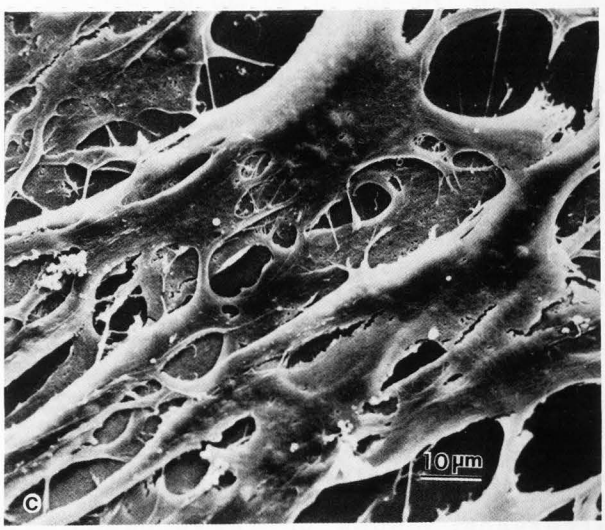
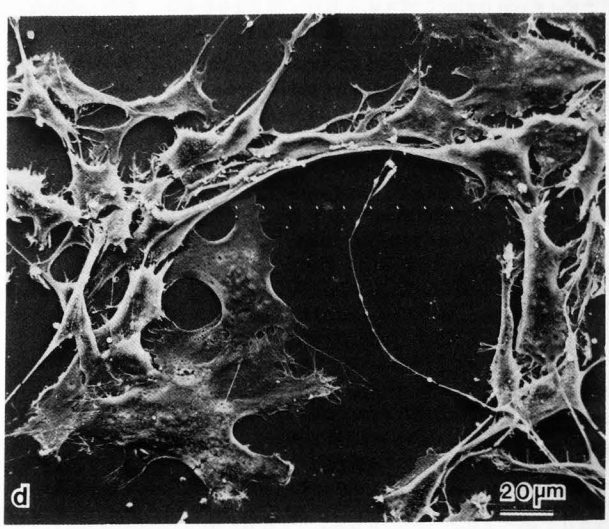
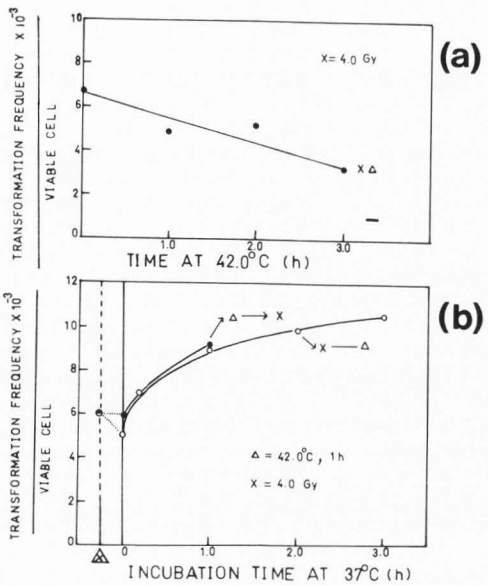


Figure 28. Scanning electron micrographs of transformed cell lines derived from C3H-10T1/2, (a) R2, (b) R7, (c) R3, (d) R25.









Figures 29a&b. Transformation frequency for C3H-10T1/2 cells exposed to heat and/or radiation. (a) Cells were heated for various times after a 4.0 Gy x-ray dose. (b) incubation at 37°C between heat and radiation or radiation and heat treatments. The transformation level from x-rays alone was 6.5 x 10^{-3} while heat treatment alone produced no transformation. The Δ represents heating.

malignant state (Raaphorst et al., 1980, 1981). Mouse embryo cells developed by Reznikoff et al., (1973) were used and the methods used to measure transformation have been described previously (Raaphorst et al., 1980, 1981). Figures 29a,b show the results of a transformation experiment involving heat and radiation. Heat treatment alone did not induce transformation at 42.0°C and other temperatures tested (Raaphorst et al., 1980, 1981). These results are supported by data from others (Harisiadis et al., 1980, Clark et al., 1981b). For combined heating and irradiation, there was a decrease in transformation frequency when heat was given immediately after irradiation (Figure 29a) and there was no significant increase in transformation for heat given immediately before or during irradiation (Figure 29b). When irradiation and heating were separated by incubation at 37°C there was a small increase in transformation frequency. This may be due to the action of an error prone repair system (Raaphorst et al., 1980, 1981). Thus, hyperthermia appears to be a non-carcinogenic agent for cancer therapy and only slightly increases the carcinogenic potential of x-rays for certain sequences of application, while providing significant enhancement of radiation cell killing.

Summary

The in vitro data show that hyperthermia and x-rays can act in a complementary fashion in terms of cell killing and in this manner radiation resistant cells in tumors might be eradicated by heating. Furthermore, combined treatment of heat and radiation resulted in a synergistic effect in cell killing. The interaction of heat and x-rays can be influenced by such factors as hypoxia, pH, nutrients, cell cycle, repair, thermotolerance, temperature, dose rate and sequence of treatments. All these factors must be considered and optimized in order to apply hyperthermia in the clinic with the greatest probability of success. For the application of hyperthermia in vivo, additional consideration must be given to the fact that many physiological factors may change thermal sensitivity or modulate the above mentioned factors. There is a great deal of work still to be done in comparing the response of cells treated in vitro to cells in tissues. In such studies the evaluation of ultrastructural and morphological changes produced by heat and/or radiation in cells in tissues may provide a physical measure of tissue damage and response.

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Discussion with Reviewers

J.R. Lepock: What might be the effect of osmotic factors in relation to work by several groups demonstrating no changes in intracellular Na^+K^+ or Cl^- content during heating and the study by Vidair and Dewey (Radiat. Res. 105, 187, 1986) showing no volume changes?

Authors: The question regarding thermally induced ion changes is still open and is discussed in the paper you refer to. That paper also indicates a decrease in volume of CHO cells heated at 45.0°C in the attached state. Our earlier data show that CHO cells heated at 42.0°C in suspension undergo little or no volume change while anisotonic extracellular milieu caused thermal sensitization (Raaphorst and Dewey, J. Therm. Biol. 3, 177-184, 1978). Thus cellular ion and volume changes due to hyperthermia may depend on mode of cell culture, heating temperature and probably other factors which need to be further examined.

J.R. Lepock: What is the contribution of cell cycle redistribution to radiosensitization in thermotolerant cells after long periods of heating at 42.0°C?

Authors: Our own results (unpublished) and the earlier studies of Sapareto et al., (Cancer Res. 38, 393-400, 1978) and Fox et al (Radiat. Res. 104, 429-442, 1985) show that during heating at 42.0°C little or no cell cycle progression occurs during the first 12 hours. Longer heating resulted in cell progression which resulted in a loss of thermotolerance and such changes would then affect radiosensitivity.

Z. Somosy: Heat treatment is known to elicit changes in the cell membrane; such as, a decrease in negative change (Sato et al., Cancer Res. 41, 4107-4110, 1981), altered permeability (Ruifrok et al, Radiat. Res. 109, 303-309, 1987) and decreased cell attachment (Anderson et al., Int. J. Radiat. Biol. 46, 399-407, 1984), similar to those observed after ionizing radiation. The question arises whether these changes contribute, and if so, to what extent to the synergism of the cell destructive effect of combined heat and radiation treatment.

Authors: As you point out, heat produces a number of membrane alterations that are similar to those produced by ionizing radiation; however, this does not mean that the membrane alterations are the cause of cell death after hyperthermia. Indeed, in the work of Ruifrok et al., (1987) and Anderson et al., (1984), the membrane alterations did not correlate with survival. Electrophoretic mobility decrease (Sato et al., 1981) and bleb formation in Gl cells (Borelli et al., 1986 text reference), correlate with heat-induced cell death; however, they are not necessarily the cause. There may be incidental effects which appear in parallel with another heat-induced change that causes cell death or the membrane changes may be another manifestation of impending death. We do not believe that membrane changes induced by heat and ionizing radiation

interact synergistically to cause cell death because sequence data, such as that shown in Figures 21 and 22, and Raaphorst et al., 1983 (text reference), show survival levels depend upon the order of heat and radiation application; and because the levels of x-ray and heat-induced killing are different through the cell cycle, (Figure 11). If cell death was due to the addition of membrane damage, the sequence of application or cell cycle position should not make a difference. In addition post treatment exposure to anisotonic treatment causes potentiation of radiation damage but not of heat damage demonstrating the different nature of the damage (Raaphorst and 61, 95-97, 1982).

T.M. Seed: All the work described employed the use of growing cells. Would the cellular response in terms of lethality, transformation, etc. under the dual irradiation/hyperthermic sensitization be altered if marginally cycling plateau phase cells were used?
Authors: Plateau phase cultures would have a larger proportion of cells in G_1 and thus the heat and radiation responses would be different than for exponentially growing cells (see Figure 11). Cell sensitivity to heat and radiation as a function of time after plotting and cell density have been studied and larger changes than can be accounted for by cell cycle redistribution have been observed (Raaphorst and Azzam, J Therm Biol. 8, 327-332, 1983). Reasons for these changes are not clear but alterations in cell attachment state and membrane morphology may be possible causes.

T.M. Seed: The authors comment on the very interesting observation concerning the enhanced hyperthermic sensitization with low dose rate irradiation. My question relates to the required prolongation of irradiation period (as required by the low dose rates). Is there enhanced cell transformation under such irradiation regimens? Also, would not such arising transformants under low dose irradiation be expected to respond differently to subsequent hyperthermal sensitization than similar cells irradiated acutely?
Authors: The work of Hahn et al., (Cancer Res. 40, 3328-3332, 1980) shows that low dose rate irradiation reduces cell transformation. Also, our own work shows that many of the transformants have similar heat and radiation responses as the progenitor normal cells (Figures 26, 27 and Raaphorst et al., 1985 text reference).