Mechanisms of the Synergistic Interaction between Hyperthermia and Radiation in Cultured Mammalian Cells

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Hyperthermia and radiation interact synergistically in cultured mammalian cells. Data pertaining to the subject are reviewed and mechanisms to explain the phenomena are proposed. One may involve changes in chromosomal structure at high temperatures. These changes make the chromosome less amenable for repair of radiation damage. A second mechanism consists of endonucleolytic degradation of DNA in hyperthermic cells after radiation. This may be ascribed to imbalance between degradation and resynthesis processes operating during repair of DNA damage.

INTRODUCTION

Modification of the response of mammalian cells to ionizing radiation is important for theoretical as well as practical reasons. It sheds light on the mechanisms by which radiation kills cells. Practically, improvement in radiation therapy of cancer becomes possible. This is why extensive work has been done concerning the radiobiology of cultured mammalian cells and factors affecting their radiosensitivity (see Elkind and Whitmore, for review).

As has been shown a physical factor like hyperthermia greatly enhances the response of cultured mammalian cells to X-irradiation. This finding emphasizes the need for a more quantitative approach in the efforts invested during the last sixty years to use hyperthermia as a tool in tumor control (see Suit and Shwayder, for review). We have been studying the synergistic interaction between hyperthermia and radiation as well as radiomimetic agents in mammalian cells. In this paper, mechanisms for the synergistic phenomena will be outlined on the basis of the available data in vitro. The situation in vivo is by far more complex, for response in vivo is modified by various factors, e.g. oxygen tension, which affect radiosensitivity. More data from in vitro experiments have to be accumulated before conclusions drawn from in vitro studies can be applied therapeutically.

EXPERIMENTAL

X-irradiation

The survival curve of X-irradiated mammalian cells or cells treated with radio-
mimetic agents is sigmoidal, i.e. it displays a shoulder before becoming exponential. This is interpreted as accumulation of sublethal damage before the cells become lethally affected. Incubation between fractionated exposures reveals the capacity of the cells to repair sublethal damage. If the cells are exposed to temperatures above 37°C before, during or immediately after irradiation, enhanced cell killing is observed. Use of fractionated exposures has shown that temperatures up to 41°C increase radiosensitivity mainly by reducing the capacity to repair sublethal damage. Higher temperatures increase cell killing by enhancing expression of lethal damage. The recovery of the capacity for sublethal damage is slow and takes over 24 hr at 37°C following heat treatment of Chinese hamster cells. This is consistent with the observation that the synergism between hyperthermia and radiation is lost very slowly during incubation at 37°C after heat treatment, and that the half time for repair of heat

Fig. 1. Arrhenius plots of the ratios of the survival curve slopes (D0) of Chinese hamster cells X-irradiated at various temperatures with a dose rate of 3.3 rad/min, ○; 12 rad/min, ●; 360 rad/min, △. The cells were suspended in buffer containing 20% growth medium during irradiation and maintained at the indicated temperature by immersion in water. The water temperature was held constant ±0.1°C during irradiation. Irradiation was with 250 KVp X-rays filtered with 1.0 mm Al plus 0.25 mm Cu under ambient O2 tension. Cells were plated for colony formation within 30 min after irradiation. Data from ref. 4.
damage which interacts synergistically with radiation is 8-16 hrs."

Of special interest is the dose-rate dependence of the synergistic interaction. This is shown in Fig. 1 where Arrhenius plots of the effect for three dose-rates are given. The effect is defined as the ratio of $D_0$ at 37°C to the $D_0$ at higher temperatures. ($D_0$ is the dose required to reduce survival by a factor of $1/e$ in the exponential region of the survival curve). Clearly, the magnitude of the effect is inversely related to the dose-rate, as could be expected. It should be noted that the synergism is not linearly dependent on temperature, except perhaps in a limited temperature range. This indicates that a single activation energy does not apply. The results could not be extended over a high enough temperature range to establish whether one observes the onset of a well defined structural change in a biopolymer complex, connected with a $T_m$.

Bromodeoxyuridine (BUdR), an analogue of thymidine, sensitizes cells to radiation
when incorporated into their DNA. In Chinese hamster cells it exerts its effect by reducing their capacity to incur sublethal damage at low concentrations ($3 \times 10^{-7}$M) and by enhancement of lethal damage at high concentrations. To gain further insight into the mechanism of the synergistic effect, studies were done using BUdR labeled cells. The results are consistent with inhibition of repair of sublethal damage by mild hyperthermia and enhancement of lethal damage expression above $41^\circ C$ (Ben-Hur and Elkind, in preparation). Figure 2 shows Arrhenius plots of the effect in BUdR labeled cells. Apparently, the only effect of BUdR is a slight perhaps not significant, increase of the temperature at which synergism is observed when BUdR is used at a low concentration. A log-linear dependence again is not observed.

Studies were also conducted relative to molecular events after X-irradiation as affected by hyperthermia. These have shown that initially the repair of X-ray-induced single-strand breaks in DNA is not inhibited by hyperthermia, but that the rate of repair of a DNA complex is slowed. Later on in time after irradiation endonucleolytic degradation of single and double-stranded DNA was observed in hyperthermic cells.

**Alkylating agents**

Methyl methane sulfonate (MMS) is a monofunctional alkylating agent which kills cells most probably by methylating DNA bases, mainly the N-7 position of guanine. A marked synergistic interaction between hyperthermia and MMS was found. The characteristics of this interaction are similar to those with X-rays, i.e. interference with the repair of sublethal damage up to $41^\circ C$ and enhancement of lethal damage expression at higher temperatures. On the molecular level the repair of damage to DNA complex and single-stranded DNA was found to be slower at high temperatures. Again, DNA degradation is observed after longer periods at $42^\circ C$. Inhibition of the repair of MMS-induced damage to single-stranded DNA has also been observed in human cells.

**Other deleterious agents**

Although DNA is thought to be the target molecule of killing by ionizing radiation and alkylating agents, other cell structures and macromolecules are also effected. Therefore the synergistic interaction between hyperthermia and agents that damage DNA specifically was also examined. These included ultraviolet (UV) radiation and the use of DNA precursors labeled with radionuclides, e.g., $^3$H-thymidine. In all cases hyperthermia ($42^\circ C$) resulted in a reduced shoulder and an increased slope of the survival curves.

**MECHANISMS**

The experimental data support the notion that hyperthermia interferes with the repair of sublethal damage. While little is known concerning the molecular nature of this repair process, it is most probably mediated enzymatically, as was shown for other repair processes. Proteins are usually heat labile and minor modifications increase this lability. As a result, mutations which cause enzymes that are active at
37°C to be inactive at 42°C or above can occur and temperature sensitive mutants have been isolated also from mammalian cells. Such mutants are selected against in natural populations of micro-organisms which are subject to extreme temperature variations. In mammals, which ordinarily maintain constant body temperature, mutations leading to heat labile enzymes may not have been selected against. Relative to radiation studies, temperature sensitive mutants of repair enzymes, if they have any slight advantage, are expected in time to dominate the cell population which by that time should display a synergism between heat and radiation. Since the likelihood that such mutations of repair enzymes will have—or will be connected with—any advantage for the cell is very small, alternative explanation must be sought.

The alternative for heat acting on the level of the repair enzymes or other enzymes which supply an essential factor (ATP, NADH) for repair enzymes is that heat acts on the level of the substrate for repair enzymes. This substrate is DNA whose double helix is resistant up to 60°C at least. However, in mammalian cells DNA is present in a complex form which can be isolated as chromatin. The DNA in chromatin is associated with proteins, mostly histones, and probably other cell material like nuclear membrane. In addition, tertiary and quarternary DNA structures due to supercoiling also exist in chromatin. These structures may be stabilized by the associated proteins and therefore should be more heat labile than the secondary structure of the double helix (For review of chromosomes and chromatin structure, see Huberman). It is proposed therefore that hyperthermia induces changes in the nucleoprotein structure which make it less amenable for repair of radiation damage. That radiosensitivity of mammalian cells can be increased by changes in chromosomal structure, has been shown by Dettor et al using hypertonic treatments.

The changes in structure involved with hyperthermia are not readily reversible since it takes a long period of time to recover from the effect of a heat treatment. Some of the structure may be changed irreversibly and has to be resynthesized. The observations that BUdR does not interact synergistically with heat alone or heat plus radiation (Fig. 2) are consistent with the mechanism proposed. This is because the effect of BUdR is on the secondary structure of DNA. Also consistent with interaction on the level of higher order DNA structures is the interference of heat with the repair of X-ray damage to DNA complex but not with single-stranded DNA.

Enhancement of lethal damage expression by hyperthermia (operationally defined as changes in the slope of the survival curve, D₀) can also be explained by interference with repair. In this case the repair is of potentially lethal damage which affects D₀. The same structural changes in chromatin that made it less amenable to repair of sublethal damage could affect similarly repair of potentially lethal damage. Indeed, recent experiments suggest that hyperthermia interferes with the repair of potentially lethal radiation damage. While this may well be the case, another mechanism seems to operate above 41°C. This includes endonucleolytic degradation of the DNA. Normally, the repair of DNA in irradiated cells involves both degradation and resynthesis and there are nucleases (endo and exo) specific for radiation
damage in DNA. It is suggested that hyperthermia interferes with the balance between the degradative and synthetic processes such that degradation becomes more prominent. This could simply happen if the nucleases involved had higher energy of activation than the polymerases. In addition, even under optimal conditions after the initial damage is repaired, degradative processes become evident in time. Hyperthermia may accelerate the onset of this degradation, thus enhancing the probability that it will occur before repair is complete. The ultimate result will be enhanced cell killing.

Finally, it should be noted that mathematical analysis of our experimental data has led Brannen to propose a model from which he concludes that there are at least two inactivating mechanisms.

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