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Effect of hyperthermia and doxorubicin on nucleoid sedimentation and poly (ADP-ribose) Polymerase activity in L1210 cells

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Summary. We report on the individual and combined effects of doxorubicin (DOX) and hyperthermia (HYP) on nucleoid sedimentation and poly (ADP-ribose) polymerase (PARP) activity of L1210 cells. The effects of HYP and DOX on nucleoid sedimentation (increased sedimentation) were similar and correlated with cell viability. No correlation of PARP activity with cell toxicity was evident; the activity of PARP was inhibited by HYP (42° C; 1–3 h) and stimulated by DOX (1–10 μ*M*; 30 min). The HYP-induced inhibition of PARP was actually ameliorated by simultaneous exposure to DOX. Although separate studies have previously suggested that chromatin alterations or the inhibition of PARP might play a role in the effect of HYP, the correlation of nucleoid changes (rather than PARP activity) with cell viability emphasizes the contribution of the former. Furthermore, the results suggest that the nucleoid technique may prove useful in screening potential treatment modalities.

Introduction

Hyperthermia is known to exhibit selective toxicity and lethality to cancer cells, and the synergistic effect of HYP and ionizing radiation [3, 17] as well as HYP and chemotherapeutic agents [1, 14] is well established. A molecular basis for the synergism between HYP and ionizing radiation is based on the assumption that HYP inhibits the repair of radiation-induced DNA damage [3, 7, 28]. The inhibition of DNA repair by HYP has been associated with an increased binding of protein to DNA, and the increased protein to DNA ratio correlated with cell toxicity [27, 29]. Hyperthermia treatment prior to ionizing radiation inhibited the repair of sublethal and potentially lethal DNA damage [3, 18], increased the level of initial DNA strand break damage [10], and inhibited the rejoining of radiation-induced strand breaks [6, 10, 20]. The inhibition of DNA repair correlated with cytotoxicity [7]. Since PARP plays a putative role in DNA repair [11, 19, 31], the relationship between HYP and the activity of PARP has recently been investigated. The pertinent findings include: (a) the enzyme is involved in the repair of radiation-induced DNA damage [15, 30]; (b) inhibitors of PARP enhanced the cytotoxicity of HYP [22]; (c) PARP was completely inactivated by brief (5 min) HYP treatment in cultured *Drosophila melanogaster* cells [24]; (d) HYP treatment of human melanoma cells decreased the level of PARP [33]; and (e) inhibitors of PARP increased the lethality of ionizing radiation [11, 23]. However, the role of PARP in DNA repair is less than established since unscheduled DNA synthesis may be increased by certain PARP inhibitors [2, 5, 21].

The inhibitors of PARP that have been shown to potentiate the damage of HYP are compounds not generally used clinically. The widely used anthracycline antineoplastic agents, especially the DNA intercalator DOX, has been demonstrated to produce large numbers of single-strand breaks associated with protein [26, 32]. Since DOX has been shown to mimic other effects of HYP, such as increased chromatin sedimentation [29, 35], and since DOX is clinically synergistic with HYP [1, 14], the effect of DOX, alone or in combination with HYP, on PARP activity and nucleoid sedimentation was investigated. An examination of the relationship between cell killing, alterations in nucleoid sedimentation, and PARP activity should provide additional information on the mechanisms involved as well as potentially useful clinical treatment modalities.

Materials and methods

Cultured L1210 cells, a mouse lymphoblastoma line originally obtained from Southern Research Institute (Birmingham, AL) and maintained routinely in our laboratory, were used in all studies. The effect of HYP on L1210 cells was determined with suspension cultures having an average doubling time of approximately 12 h between cell densities of 5×10^4 and 1×10^6 cells/ml.

[^{3}H]Thymidine (sp. act. 69.4 Ci/mmol) was purchased from ICN. All other chemicals were purchased from Sigma Chemical Company or Aldrich Chemical Company. The cells were labeled with 0.10 μCi/ml [^{3}H]thymidine at an initial culture density of 1×10^5 cells/ml [16]. After labeling, the cells were centrifuged and resuspended to 3×10^5 cells/ml in fresh culture media, and 2-ml aliquots were treated with HYP and/or DOX [29]. Control cells were maintained at 37° C. After treatment, aliqots of the suspensions were counted using trypan blue or assayed by bioassay in BDF_1 mice [29].

Cells to be used for nucleoid studies were washed and resuspended in phosphate-buffered saline (0.02 *M* sodium

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phosphate, pH 7.4, 0.87% NaCl, PBS) at a concentration of approximately 6×10^6 cells/ml, and the sedimentation of nucleoids was conducted [8, 16] with the following modifications. Gradients of 15% and 30% sucrose containing 1.95 *M* NaCl, 0.01 *M* TRIS, pH 8.0, and 0.001 *M* EDTA were prepared in 5.0-ml cellulose nitrate tubes, using a Buchler density gradient maker. A 0.5-ml aliquot of 60% sucrose was used as a cushion. A 75-µl aliquot of lysing solution (2.6 *M* NaCl, 0.133 *M* EDTA, 2.6 m*M* TRIS, 0.66% Triton X-100, and 0.67% Sarkosyl) was layered gently onto the gradients, to which was then added 50 µl of a suspension containing $2-4 \times 10^5$ cells/ml in PBS. A second 75-µl aliquot of lysis solution was layered over the cell suspension. After a 30 min lysis period in a dark environment, the gradients were centrifuged for 30 min at 22,000 rpm at 20° C in an SW 50.1 rotor, using a Beckman Model L-2 ultracentrifuge. Fractions of eight drops were collected on Whatman No. 1 filter paper strips, the strips were dried, and the radioactivity was counted in a Searle liquid scintillation counter.

The percentage of radioactivity in each fraction was calculated, and the distance traveled by the nucleoids was measured as the distance of the [^{3}H] peak from the top of the gradient. Relative distances were then calculated as the ratio of the distance traveled by nucleoids from treated cells to the distances traveled by nucleoids from control cells in the same centrifugation [12]; this was called the migration ratio. The sedimentation of the nucleoid peak varied from fractions 10 to 13.

The activity of PARP was measured according to previously published procedures [4]. Cells ($2-5 \times 10^6$) were centrifuged, washed twice in ice-cold PBS, and resuspended in 1 ml lysis solution (5% dextran T-110, 10 mmol/l $MgCl_2$, 0.05% Triton X-100, 30 mmol/l NAD^+ plus 0.5 Ci/ml ^{3}H-NAD^+ (New England Nuclear) and 40 mmol/l TRIS, pH 8.0). After lysis, the suspensions were incubated at room temperature for 60 min with triplicate 0.1-ml aliquots removed at timed intervals and added to ice-cold 10% TCA. Radiolabelled product was recovered on GF/C filters (Whatman), and the amount of incorporated isotope determined by liquid scintillation spectroscopy.

Results

Nucleoids isolated from HYP- and DOX-treated L1210 cells sedimented faster in sucrose density gradients than nucleoids from control cells. The results of a sample gradient are shown in Fig. 1. Although the peak widths and the peak location varied among experiments, the results were similar when expressed in relation to the sedimentation of the control nucleoid (i.e., migration ratio). For example, the average migration ratio was 1.50 ± 0.12 ($N=5$) and 1.58 ± 0.18 ($N=3$) for HYP- and DOX-treated cells, respectively. The corresponding cell viability (trypan blue) was 1.0 ± 0.14% and 0.7% ± 0.2%.

Since the alterations in nucleoid sedimentation were essentially maximal under the conditions described in Fig. 1, and since we were interested in the results of combining DOX and HYP, conditions were chosen such that very little effect on nucleoid sedimentation or cell survival was observed by either agent (i.e., 1 µ*M* DOX × 30 min +/− 42° C × 30 min). Under such conditions, HYP and DOX alone did not significantly alter the migration ratio, and cell viability was only slightly altered (Table 1). The simultaneous treatment of L1210 cells with nondamaging conditions of HYP and DOX resulted in an enhancement of the alterations in nucleoid sedimentation and cell viability (Table 1).

Table 1. Effect of hyperthermia and/or doxorubicin on alterations in nucleoid sedimentation, poly (ADP-ribose) polymerase activity, and cell viability[a]

Treatment	Migration ratio	Cell viability (%)[b]	PARP activity (relative)
30° C × 30 min	1.00	100%	1.00
42° C × 30 min	1.04 ± 0.08	95 ± 4	1.00
37° C plus DOX (1 µ*M*) × 30 min	1.10 ± 0.12	88 ± 7	0.94 ± 0.13
42° C plus DOX (1 µ*M*) × 30 min	1.48 ± 0.12	0.10 ± 0.08%[c]	1.88 ± 0.22

[a] Results are expressed as the mean ± SD of 3 to 5 experiments
[b] Cell viability given as percentage of L1210 cells unstained by trypan blue following the indicated treatment, unless otherwise indicated. For each treatment condition 500 cells were counted
[c] Bioassay in male BDF_1 mice were inoculated with 10^5 L1210 cells and the animals were followed for 30 days. Bioassay resulted in 100% (10/10) of the animals surviving longer than 30 days

The observed enhancement with DOX and HYP, together with the demonstration that HYP inhibited PARP activity and that inhibitors of this enzyme in combination with HYP resulted in enhanced cytotoxicity [22], prompted an examination of the effects of DOX and HYP on the activity of PARP in L1210 cells and a study of the relationship of PARP activity to nucleoid changes. Hyperthermia inhibited the activity of PARP (Fig. 2) and the inhibition was a function of exposure time (1–3 h at 42° C). Although DOX and HYP produced similar changes in nucleoid sedimentation, exposure to DOX resulted in a marked stimulation of PARP activity in a dose-dependent fashion

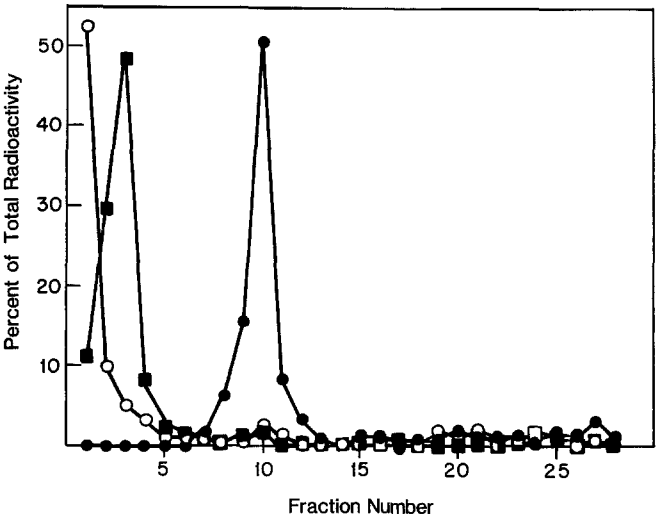

Fig. 1. Sedimentation profile of nucleoids from control and treated L1210 cells in neutral sucrose gradients. Cells were labelled with [^{3}H] thymidine at 37° C. ●, control; 0, 42° C × 3 h; ■, 10 µ*M* doxorubicin × 30 min. Sedimentation is from right to left

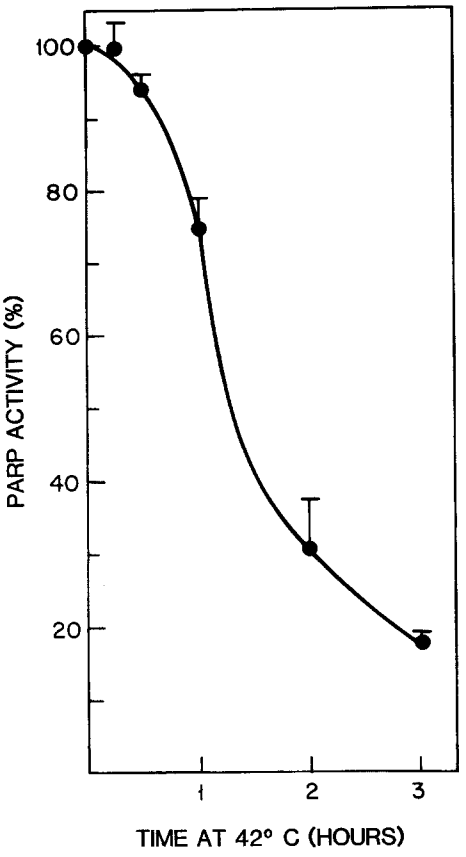

Fig. 2. Poly (ADR-ribose) polymerase activity as a function of duration of hyperthermia treatment at 42° C

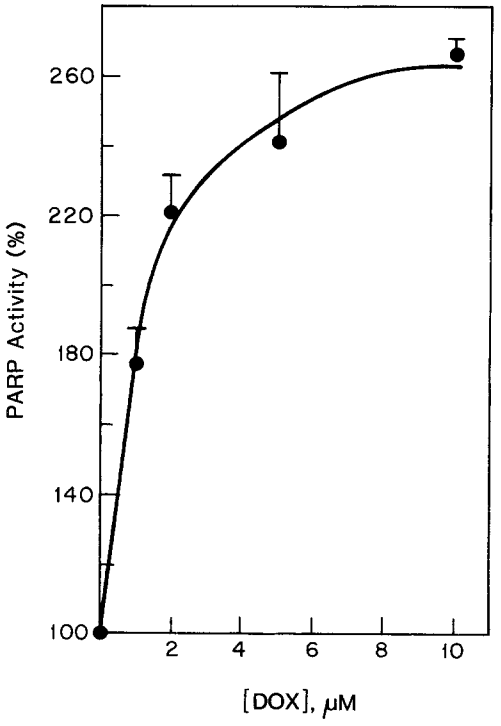

Fig. 3. Poly (ADP-ribose) polymerase activity as a function of the concentration of doxorubicin. Doxorubicin exposure was for 30 min

(Fig. 3). Furthermore, the simultaneous presence of DOX and HYP actually increased the activity of the enzyme while killing >99% of the tumor cells (Table 1).

Discussion

The nucleoid sedimentation assay was chosen because it is the most sensitive assay for DNA breaks [8, 9] and provides a means of studying the repair of DNA lesions induced by γ-radiation or DNA-damaging chemicals [12, 25]. The DNA repair mechanism has received considerable attention as an explanation of the synergism between HYP and ionizing radiation. The model chosen was based on the sensitivity of the nucleoid assay to DNA breaks and also on the ability of the nucleoid technique to detect an increased protein content of nucleoids; this ability was viewed important in further understanding the role of DNA repair inhibition by HYP, since considerable data suggest that alterations in the association of proteins with DNA may be involved in the inhibition of DNA repair [27]. Furthermore, DOX has been reported to cause an increased sedimentation of chromatin [35] similar to that reported for HYP.

It is well established that PARP activity is increased when DNA contains strand breaks induced by alkylating agents [30, 31], UV irradiation [30, 31], or DNase I [31]. Treatment of L1210 cells with HYP significantly decreased PARP activity; the activity of the enzyme from *Drosophila* was abolished [24]. The enzyme from L1210 cells more closely resembles the enzyme from human melanoma cells in terms of the effect of HYP on enzymic activity [33].

Poly (ADP-ribose) polymerase is a heat-labile, chromatin-bound enzyme that catalyzes the covalent attachment of homopolymers of ADP-ribose (derived from NAD^+) to receptor molecules (the enzyme itself, histones, nonhistone nuclear proteins) [13]. The association of proteins with DNA, and their possible involvement in DNA condensation reactions and transcription and replication, as well as DNA repair, is related to the reversible chemical modification of these proteins [34]. Poly (ADP-ribose) polymerase results in a chemical modification associated with DNA repair processes [11, 19, 31]. However, controversy exists since some inhibitors of PARP stimulate repair in some systems [2, 5, 21].

The results indicate that DOX-induced and HYP-induced nucleoid changes closely paralleled the cytotoxic effects of each treatment modality alone and when HYP and DOX were administered together. However, PARP activity did not correlate with cell toxicity. Although a 5-min heat shock completely inactivated the PARP of cultured *Drosophila melanogaster* cells, there was no significant effect on the growth of the cells [24]. These results argue against the importance of DNA damage that would be repaired by PARP in cell toxicity and favor the importance of DNA damage exemplified by alterations in nucleoid structure. The lack of correlation of PARP activity with cell survival and the correlation of nucleoid changes with cell survival suggest that: (a) inhibition of PARP and presumably of DNA repair is not the primary mechanism of action of HYP; and (b) HYP and DOX induced similar damage to nucleoids, and the correlation with cell survival suggests a role for nucleoid alterations in the mechanism of action of each treatment modality alone or in combination. The results also argue against PARP being involved

in the nucleoid alterations produced by ADR of HYP. The importance of these observations demonstrate the need to determine the synergistic potential of treatment modalities, not by their ability to cause DNA damage evidenced by strand breaks, but by their ability to cause DNA damage resulting from alterations in nucleoid sedimentation. Studies should address the nature of the nucleoid alterations with attention toward characterization of the nature of the increased protein and the nature of the DNA damage induced by various DNA-damaging agents.

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