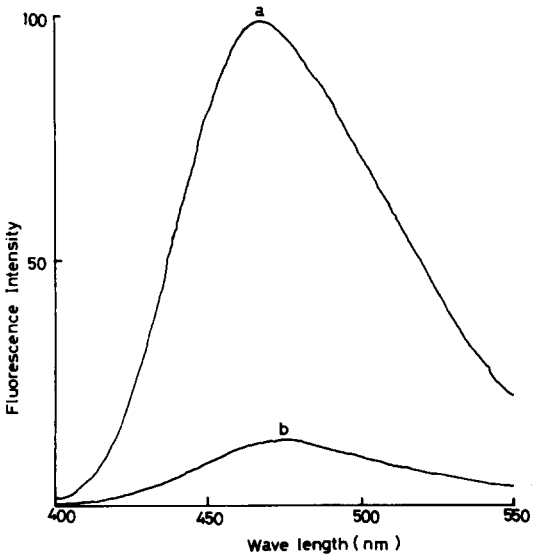

Fig. 5. Emission spectra of ANS in RSA (a) and FrII (b) solutions. ANS concentration was 20 μM and protein concentrations were adjusted to 300 μg/ml in both solutions. Spectra were measured at 380 nm for excitation. Fluorescence intensities were expressed by relative values.

In conclusion, ANS can be used as a convenient probe to detect anionic drug binding protein in muscle, and the ANS titration method is a convenient method to determine the binding constant of anionic drugs. We are now proceeding to purify FrII.

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Effect of 1-β-D-arabinofuranosyl cytosine and hydroxyurea on the repair of X-ray-induced DNA single-strand breaks in human leukemic blasts*

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We have demonstrated previously that ara-C incorporates into DNA of murine and human leukemic cells [1, 2]. The extent of ara-C incorporation into DNA correlates with inhibition of DNA synthesis, and the ara-C residue behaves as a relative chain terminator [3]. These findings are consistent with conformational and hydrogen bonding differences of the arabinose sugar moiety altering reactivity of the 3′-terminus and slowing chain elongation [4, 5]. This disruption of DNA replication by ara-C results in DNA fragmentation [6].

Ara-C inhibits repair of DNA damage of the type induced by u.v. light and certain alkylating agents [7–10]. However, the precise mechanism by which ara-C inhibits repair has not been defined at a molecular level. Ara-C has been shown to inhibit repair of u.v.-induced DNA strand breaks [8]. We recently demonstrated that ara-C incorporates into DNA undergoing repair of u.v. damage [11]. Furthermore, the extent of ara-C (DNA) formation during u.v. repair correlates significantly with cell lethality [11]. These findings thus demonstrated that ara-C incorporates into DNA undergoing large patch repair [12, 13] induced by u.v. irradiation. Since large patch DNA repair synthesis would increase the frequency of ara-C misincorporation, it was of interest to determine whether other agents, such as X-ray, which induce short patch DNA repair would be similarly inhibited by ara-C.

The present study has monitored the effects of ara-C alone and in combination with hydroxyurea on the repair of X-ray-induced single-strand breaks. The results demonstrate that these agents partially inhibit this repair process. However, in contrast to our previous findings with u.v. irradiation, the inhibition of X-ray-induced DNA repair is not associated with detectable incorporation of ara-C. These findings may be relevant when considering therapeutic approaches that employ ara-C in combination with agents that damage DNA.

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Materials and methods

Cells. The human leukemic blasts were maintained in suspension culture as previously described [14]. Chromosomal analysis revealed a human 45 × $^{-}9^{+}$ karyotype.

Alkaline elution analysis. The human leukemic blasts were labeled with 0.01 μCi/ml [^{14}C]thymidine (52 mCi/mmole; New England Nuclear, Boston, MA) for 24 hr, washed, and then irradiated as a pellet on ice with 3 or 10 Gy X-ray. Cells were allowed to repair at 37° for 30 min or 1 hr. The effects of 10^{-3} M ara-C (Sigma Chemical Co., St. Louis, MO) alone or in combination with 2×10^{-3} M hydroxyurea (Sigma) were determined by treatment of the human leukemic blasts for 0–2 hr at 37° before X-irradiation on ice and then during repair at 37° for 1 hr. Internal standard cells were labeled with 0.1 μCi/ml [^{3}H]thymidine (2 Ci/mmole; New England Nuclear), irradiated, amd maintained on ice until performing the alkaline elution analysis. The alkaline elution technique and the calculation of single-strand break frequency have been described previously [6, 15]. Relative retention values were calculated for ^{14}C-labeled DNA at 50% elution of ^{3}H-internal standards treated with 3 Gy plotted according to a decreasing exponential function using the Marquardt/Levenberg curve fitting method of the MLAB computer program [16].

Incorporation of [^{3}H]*ara-C into DNA undergoing repair synthesis.* The human leukemic blasts in logarithmic growth phase were incubated for 2 hr in the presence of 10^{-5} M BrdUrd and 10^{-6} M FdUrd. Cells were then treated with 2×10^{-3} M hydroxyurea for 30 min, irradiated with 10 Gy, and incubated with 10^{-6} M [^{3}H]ara-C (15.5 Ci/mmole; Radiochemical Centre, Amersham, England) for 1 hr at 37°. The DNA was extracted after lysis with 2.5 mg pronase B and 0.01 M Tris (pH 7.4), 0.01 M EDTA, and 0.5% sodium dodecyl sulfate [1]. The nucleic acids were precipitated with 4 M NaCl and ethanol. DNA was then isolated on CsCl gradients as previously described [17].

Results

We have demonstrated previously that exposure of leukemic cells to ara-C results in accumulation of strand breaks in DNA undergoing semiconservative and unscheduled DNA synthesis [6]. These effects were dependent upon duration of drug exposure. Thus, total duration of drug treatment in the present study on human leukemic blasts was limited to a 2-hr incubation with 10^{-3} M ara-C prior to 3 Gy X-irradiation. The ara-C exposure was then continued during a 1-hr period of DNA repair before analysis by alkaline elution. Under these experimental conditions, there was no significant inhibition of the repair of X-ray-induced DNA single-strand breaks.

The potentiation of hydroxyurea of the effects of ara-C on DNA repair [18] prompted the monitoring of this combination on the repair kinetics of X-ray-induced damage. Figure 1 illustrates the effects of 10^{-3} M ara-C and 2×10^{-3} M hydroxyurea on the repair of single-strand breaks induced by 3 Gy. The rate of DNA elution was identical for cells treated with 3 Gy alone and in combination with hydroxyurea/ara-C during the 1-hr recovery (Fig. 1A). A 1-hr preincubation with hydroxyurea/ara-C resulted in an apparent inhibition of the kinetics of 3 Gy DNA repair. However, the combination of hydroxyurea/ara-C alone increased the rate of DNA elution and thus did not actually inhibit the repair kinetics of X-ray-induced damage (Fig. 1B). Similar findings were obtained with a 2-hr incubation with hydroxyurea/ara-C prior to 3 Gy X-irradiation (Fig. 1C). Although the 1-hr and 2-hr preincubations would conceivably enhance ara-CTP pool formation, the rate of DNA elution from cells treated by 3 Gy in combination with hydroxyurea/ara-C was thus not significantly greater than the additive effects of 3 Gy alone and hydroxyurea/ara-C alone.

Further attempts at detecting inhibition of X-ray repair by hydroxyurea/ara-C were performed by employing 10 Gy irradiation and shorter drug exposure. The repair of single-strand breaks induced by 10 Gy was nearly complete after 45 min of incubation at 37° (data not shown). The leukemic blasts were thus exposed to 2×10^{-3} M hydroxyurea for 30 min, and 10^{-3} M ara-C was added just prior to 10 Gy X-irradiation. The blasts were then allowed to undergo repair at 37° for 30 min. A representative profile is illustrated in Fig. 2. Under these experimental conditions, the elution rate of ^{14}C-labeled DNA from untreated cells is compared with that of a reference ^{3}H-labeled DNA from untreated cells. Using this approach, treatment with ara-C or with hydroxyurea/ara-C increased the number of single-strand breaks by 38 and 47%, respectively, as compared to that obtained after repair of 10 Gy alone. The strand break frequencies are included in the legend to Fig. 2. Thus, ara-C alone and hydroxyurea/ara-C exposure can result in partial inhibition of repair of X-ray-induced DNA strand breaks.

Our previous studies have demonstrated that ara-C incorporates into DNA undergoing repair of u.v.-induced damage [11]. Similar experiments were performed in the present study to assess the incorporation of [^{3}H]ara-C into DNA undergoing damage induced by 10 Gy over a 1-hr period at 37°. Although this approach would be sensitive to 0.01 pmole ara-C incorporated into DNA per 10^{6} cells, there was no detectable incorporation of [^{3}H]ara-C into leukemic cell DNA undergoing this repair (data not shown).

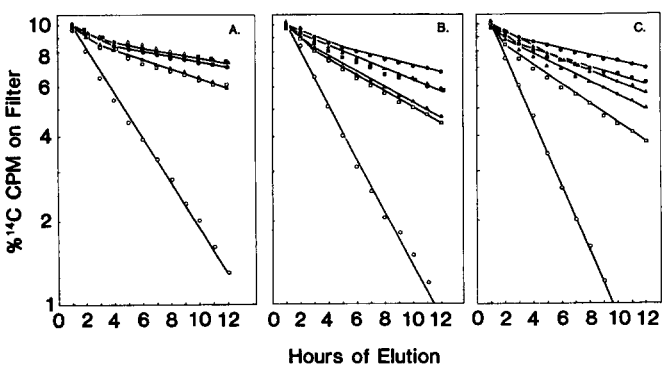

Fig. 1. Effects of 10^{-3} M ara-C and 2×10^{-3} M hydroxyurea/10^{-3} M ara-C on repair of 3 Gy X-ray-induced DNA strand breaks. Human leukemic blasts in logarithmic growth phase were exposed to drug for 0 min (A), 60 min (B) or 120 min (C) at 37° prior to treatment with 3 Gy at 0°. The cells were then incubated with or without drug for an additional 1 hr at 37° before analysis by alkaline elution. Key: no treatment control (●); 3 Gy X-ray (no recovery) (○); 3 Gy X-ray (1-hr recovery) (▲); hydroxyurea/ara-C (■); ara-C and 3 Gy X-ray (1-hr recovery) (△); and hydroxyurea/ara-C and 3 Gy X-ray (1-hr recovery) (□).

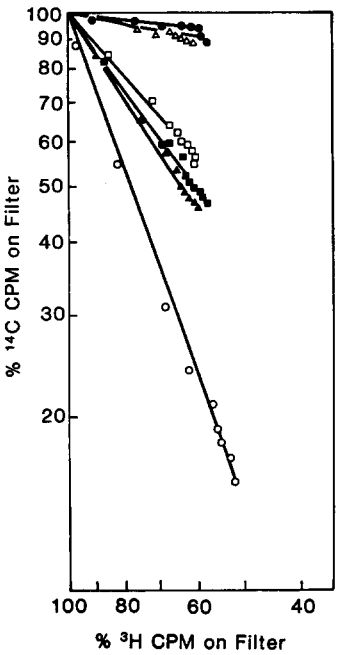

Fig. 2. Effects of 2×10^{-3} M hydroxyurea/10^{-3} M ara-C on repair of 10 Gy X-ray-induced DNA strand breaks. Human leukemic blasts in logarithmic growth phase were exposed to hydroxyurea for 30 min, and then ara-C was added just prior to irradiation with 10 Gy at 0°. The cells were then incubated in drug for an additional 30 min at 37° before analysis by alkaline elution. Symbols represent: no treatment control (●); 10 Gy X-ray (no recovery) (○); 10 Gy X-ray (30-min recovery) (□); hydroxyurea/ara-C (△); ara-C and 10 Gy X-ray (30-min recovery) (□); hydroxyurea/ara-C (△); ara-C and 10 Gy-X-ray (30-min recovery) (■); hydroxyurea/ara-C and 10 Gy X-ray (30-min recovery) (▲). DNA single-strand breaks/10^7 nucleotides (mean ± S.D.) were: 10 Gy X-ray (30-min recovery): 0.908 ± 0.005; hydroxyurea/ara-C: 0.003 ± 0.001; ara-C and 10 Gy X-ray (30-min recovery): 1.251 ± 0.007; hydroxyurea/ara-C and 10 Gy X-ray (30-min recovery): 1.332.

Discussion

We have recently used the alkaline elution technique to demonstrate that ara-C induces strand breaks in DNA undergoing semiconservative and unscheduled synthesis [6]. Ara-C is also a potent inhibitor of repair of DNA damage induced by u.v. light and certain alkylating agents [7–10]. However, the mechanism by which ara-C inhibits repair has not been defined at the molecular level. We have demonstrated that ara-C incorporates into DNA undergoing repair of u.v.-induced damage and that the extent of this incorporation correlates with loss of clonogenic survival. These findings are in concert with our previous observations regarding the effect of ara-C incorporation on inhibition of semiconservative DNA synthesis and induction of lethal cellular events [1–3].

DNA repair has been divided into long and short patch processes. The long patch repair of u.v. damage is thought to occur by excision of approximately 100 nucleotides and then resynthesis steps [13]. In contrast, short patch repair occurs following DNA damage with X-irradiation [13]. Ionizing radiation induces multiple DNA lesions, including single- and double-strand breaks, base-free sites and modification of DNA bases [19]. The patch size for repair of ionizing radiation damage is approximately 3–4 nucleotides based upon experiments monitoring thymidine incorporation [13]. Thus, the incorporation of ara-C would be dependent upon a requirement for deoxycytidine in the repair patch. Hydroxyurea potentiates the effects of ara-C on DNA repair, presumably by decreasing dCTP pools [18].

The present study demonstrates that hydroxyurea and ara-C partially inhibit the repair of X-ray-induced DNA single-strand breaks. These results are consistent with a recent finding in HeLa cells [20]. Although greater inhibition of repair by 10^{-4} M ara-C or 10^{-2} M hydroxyurea was noted in that study, such differences are probably methodological. Thus, the inhibition of HeLa cell repair was most pronounced within 5 min after the induction of DNA damage and then declined to about 30% by 30–60 min. Those experiments also monitored repair kinetics at 24°. The differences in inhibition of X-ray-induced repair may also vary with such parameters as duration of drug exposure and irradiation dose. In this regard, we were unable to demonstrate significant drug inhibition of DNA repair following a dose of 3 Gy while partial inhibition of this repair was observed after a dose of 10 Gy. Furthermore, the time- and concentration-dependent accumulation of DNA fragments by ara-C had to be taken into account when assessing the inhibitory effects of this agent on DNA repair [6]. Thus, treatment with 10 Gy X-irradiation combined with a shorter exposure to hydroxyurea and ara-C permitted the detection of a partial inhibition of repair without the induction of extensive single-strand break accumulation by these drugs alone.

DNA repair after X-irradiation was not accompanied by a detectable incorporation of [^{3}H]ara-C in the repair patch as we had previously demonstrated during repair of u.v.-induced damage [11]. This finding may be related to the small repair patch associated with X-ray induced damage and thus the lower probability of ara-C misincorporation. Furthermore, the difference in incorporation of ara-C into DNA undergoing repair synthesis of u.v.-induced damage as compared to X-ray-induced damage could be related to the polymerase involved in these repair processes. Semiconservative synthesis and long patch repair utilize polymerase α, while short patch repair synthesis probably involves polymerase β [10]. In contrast to DNA polymerase α, DNA polymerase β is inhibited only by high concentrations of ara-CTP. Our present and previous findings may thus be explained by differences in the capacity of DNA polymerases α and β to incorporate ara-C into DNA and/or excise [21, 22] these residues from the 3′-terminus. This distinction may be important when attempting to combine ara-C with therapeutic agents that damage DNA by mechanisms which would result in either long or short patch repair processes.

In summary, we have demonstrated previously that ara-C incorporates into leukemic cell DNA. The extent of (ara-C)DNA formation correlates with inhibition of semiconservative DNA synthesis. Ara-C also inhibits the repair of DNA damage induced by u.v. light and misincorporates in the long repair patch. The present study using alkaline elution analysis has monitored the effects of ara-C on repair of X-ray-induced DNA damage in human leukemic blasts. Ara-C alone and in combination with hydroxyurea was ineffective in inhibiting 3 Gy X-ray-induced repair. However, a partial inhibition of the repair of damage induced by 10 Gy X-ray was observed with these drugs. In contrast to our previous findings with u.v. irradiation, the inhibition of X-ray-induced DNA repair was not associated with detectable incorporation of ara-C. These findings may be related to the small size of the DNA repair patch or the polymerases involved in the different repair processes. The present results could be relevant when considering therapeutic approaches that employ ara-C in combination with agents that damage DNA.

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Distinctive responses of heart muscle and non-muscle cells to oxygen and glucose deprivation as regards phospholipid fatty acids

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Heart cells in culture allow metabolic and pharmacological investigations to be performed excluding the systemic reactions and interactions challenged in the whole heart by experimental conditions and environmental factors [1].

Moreover, they offer the possibility of distinguishing the particular responses between muscle and non-muscle cells, which coexist in heart tissue, by growing them separately. Compared to non-muscle cells, one of the distinctive features of muscle cell and mixed cell cultures is their beating activity, which is correlated to a higher level of β-oxidation [2].

In the last few years, cardiac cell cultures served also as an experimental model to several authors who studied the effects of oxygen deprivation upon heart tissue (see for instance [1–7]). In our laboratory, we considered fatty acid oxidation and non-esterified fatty acid composition [8], energy metabolism [9], hypoxanthine and enzyme leakage [10]. The present work was designed to characterize the ischemia-induced modifications in membrane phospholipids in the same preparation.

Rat heart cells were cultured either mixedly (H cells) or separately (M = muscle cells, F = non-muscle cells). Ischemia was mimicked by imposing simultaneously to the cultures three conditions related to it: partial oxygen deprivation, deficiency of energy-yielding substrates, especially glucose, and accumulation of metabolic end-products. Phospholipids were scrutinized with regard to their fatty acid composition.

Materials and methods

Heart cell cultures. These were prepared from 3-day-old rats (Sprague–Dawley) according to Harary and Farley [11] with some modifications. Cells were isolated from the minced hearts by trypsinization at 23°. The enzyme, twice crystallized from pig pancreas (Labor. Choay, Paris) was used at a 0.5–1.0 g/l concentration in Dulbecco's buffered Ca^{2+}- and Mg^{2+}-free salt solution. Repeated 5-min incubations with trypsin were done until the tissue was almost completely dispersed. To obtain mixed cell cultures (H), the cells were plated into Corning culture flasks at a density of $1.2.10^5/cm^2$ in Eagle's minimum essential medium (MEM) (4.2 ml/1.10^6 cells) containing Earle's salts, glucose 5.55 mM, antibiotics and 10% calf serum. Muscle and non-muscle cells were separated by means of a differential attachment technique, based on the fact that non-muscle cells attached faster to the substrate of the culture flask. To establish F cultures, the trypsinized cells were plated at a density of $3.2.10^5/cm^2$ in MEM (1.1ml/1×10^6 cells) and incubated at 37°. Thirty-five to 40 min later, F cells interspersed with 5–10% M cells were attached to the flask. The medium, which contained floating M cells interspersed with 10% F cells, was recovered, replaced in F cell flasks by fresh medium and centrifuged. The cell pellet (M cells) was resuspended in MEM and plated at a density of $1.61.10^5/cm^2$ in MEM (3.1 ml/1.10^6 cells). M, F and H cell cultures were grown for 6 days at 37°. The gas phase consisted of air without addition of CO_2. The pH was adjusted every