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Effects of Hemin on Apoptosis, Suppression of Cytochrome C Oxidase Gene Expression, and Bone-Marrow Toxicity Induced by Doxorubicin (Adriamycin)

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ABSTRACT. We have shown that hemin (iron-protoporphyrin IX) selectively counteracts doxorubicin (Adriamycin, ADR)-induced cytotoxicity on human leukemia K-562 cells by preventing ADR from inhibiting mitochondrial cytochrome c oxidase (COX), a novel target site for anthracyclines. Here, we investigated whether or not (a) treatment with ADR promotes apoptosis and represses the expression of two COX genes (one nuclear and one mitochondrial) in human K-562 cells in the absence and presence of hemin, and (b) injection of hemin preserves bone-marrow cellularity in ADR-myelosuppressed rats. Cultured K-562 cells were incubated with varying concentrations of ADR.HCl (0.2 μM to 5 μM) in the presence and absence of hemin (30 μM) and assessed for DNA degradation, as well as for expression of mitochondrial COXII and nuclear COXIV genes by RNA Northern blot hybridization analysis. In parallel, we investigated whether or not hemin injected i.p. in myelosuppressed rats affected ADR-induced bone-marrow cytotoxicity. These studies have shown the following: (a) ADR caused a dose- and time-dependent DNA fragmentation, characteristic of apoptosis, in K-562 cells; (b) hemin reduced the frequency of cell death caused by ADR: this effect was specific for ADR, because hemin failed to prevent apoptosis induced by methotrexate (MTX) in these cells; (c) ADR suppressed expression of COXIV and COXII genes, and exposure of ADR-treated K-562 cells to hemin did not reverse this suppression; and (d) i.p. injection of hemin in ADR-myelosuppressed rats improved bone-marrow cellularity, promoted colony formation (CFU-GM and CFU-F), and stromal cell outgrowth; moreover, hemin increased WBC counts depressed 12 days after ADR treatment. These studies indicate that hemin is a selective inhibitor of ADR-induced apoptosis of human leukemia cells and preserves bone-marrow cellularity in rats injected with ADR. BIOCHEM PHARMACOL 52;5:713–722, 1996.

KEY WORDS. doxorubicin; adriamycin; hemin; human K-562 cells; apoptosis; cytochrome c oxidase genes; myelosuppression

ADR† and analogues are potent antineoplastic agents with wide clinical applications in a large variety of human cancers. However, their long-term clinical use is limited due to severe bone-marrow suppression and cumulative dose-dependent cardiovascular toxicity. Unfortunately, the precise mechanisms by which ADR causes myelosuppression and cardiovascular toxicity are not fully understood. The fact that bone marrow consists of several hemopoietic progenitors and stromal cells (fibroblasts, endothelial cells, adipocytes, and macrophages) that interact with each other *via* growth factors [1] has made it difficult to determine whether or not ADR preferentially kills a given cell population vs another in bone marrow (i.e. stromal fibroblastoid cells or hemopoietic progenitors or both), resulting in myelosuppression.

In recent years, a number of different mechanisms have been proposed to explain anthracycline-induced cytotoxicity: (a) direct interaction with the genome [2]; (b) interaction with plasma membrane phospholipids [3]; (c) promotion of lipid peroxidation [4]; (d) free radical formation [5]; (e) stabilization of the DNA-topoisomerase cleavage complex [6]; and (f) deterioration of mitochondrial structural and functional integrity [7–9]. In addition, like most chemotherapeutic agents, anthracyclines can induce programmed cell death (apoptosis) [10]. Apoptosis is characterized by cytoskeleton disruption, cell shrinkage, alter-

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† *Abbreviations:* ADR, adriamycin; DAU, daunomycin; MTX, methotrexate; COX, cytochrome c oxidase; WBCs, white blood cells; BMNCs, bone marrow nucleated cells; LTBMCs, long-term bone marrow cultures; CFU-GM, granulocyte-macrophage colony forming unit; CFU-F, fibroblast-colony forming unit.

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ations in membane permeability, and a nonrandom degradation of nuclear DNA either into oligonucleosome-sized fragments or into larger DNA fragments, depending on specific cell types.

We suggested earlier that ADR may cause a dual inhibitory action. On the one hand, it could inhibit biosynthesis of COX subunits at the transcriptional level and, on the other, inhibit COX activity directly [9]. The observation that apoptosis has been shown to involve mitochondrial impairment [11] and that mitochondrial COX serves as a novel target of ADR [9] prompted us to examine whether or not: (a) ADR-induced cytotoxicity of leukemia cells is mediated by apoptosis; (b) ADR represses the expression of COX genes in a way that is affected by hemin; and (c) hemin, an agent that prevents ADR-cytotoxicity on human leukemia K-562 cells, prevents apoptosis as well as bone-marrow cytotoxicity induced by ADR.

MATERIALS AND METHODS

Chemical and Biological Materials

Hemin (iron-protoporphyrin IX) was purchased from Eastman Kodak (Rochester, NY, U.S.A.), dissolved in slightly alkaline solution and sterilized by using a 0.44 μm Millipore filter. RPMI-1640 medium, McCOY's 5a medium (modified, without serum, with L-glutamine), fetal calf serum (FCS), and horse serum, were all purchased from GIBCO, Life Technologies, Inc. (Paisley, Scotland). Doxorubicin (Adriamycin) hydrochloride, LiCl, NaCl, MOPs (3-[N-Morrpholino]-propane-sulfonic acid), L-glutamine 200 mM (100 ×) and L-serine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Urea was purchased from Mallinckrodt, Inc. (St. Louis, MO, U.S.A.). Ultra-pure agarose was purchased from Mallinckrodt, Inc. (St. Louis, MO, U.S.A.). Ultra-pure agarose was purchased from BRL, Life Technologies Inc., (USA). Hybridization transfer membranes HYBOND-N, Multiprime DNA Labelling System and radioisotope [a-^{32}P]-dCTP were purchased from Amersham, U.K. Penicillin-streptomycin solution (10,000 units of penicillin and 10,000 μg streptomycin/mL) in saline, MEM amino-acid solution (50 ×) without glutamine, MEM nonessential amino acids (100 ×), MEM vitamin solution (100 ×), sodium pyruvate MEM 100 mM, lyophilized beef embryo extract (BEE), bovine citrated plasma (BCP) and L-asparagine. H_2O (ASP) all were purchased from GIBCO. May-Grunwald and Giemsa solutions were purchased from E. Merck (Darmstadt, Germany). Adriblastina (ADR.HCl), purchased in the form of lyophilized powder from Farmitalia Carlo Erba (Italy), was reconstituted prior to injection. MTX sodium was also purchased in the form of lyophilized powder from Lederle (U.S.A.). Solu-Cortef (hydrocortisone sodium succinate) was a product of Upjohn (Puurs, Belgium). Topostatine, purchased as lyophilized thrombine (3000 units N.I.H.) from Roche (Basel, Switzerland), was used to speed up coagulation of plasma clots. Conditioned medium (5637-CM) enriched in GM-CSF, G-CSF, IL-1, and IL-6 growth factors was obtained from confluent cultures of bladder carcinoma cell line 5637 after centrifugation at 600 × *g* for 5 min [12] and stored at 4°C. PBS containing NaCl (8 g/L), KCl (0.2 g/L), $Na_2HPO_4.2H_2O$ (1.15 g/L) and KH_2PO_4 (0.2 g/L) was prepared in our laboratory. Restriction enzymes were purchased from Minotech (Crete, Greece).

Cell cultures

Human K-562 erythroleukemia cells, originally developed by Lozzio and Lozzio [13], were seeded in culture at a concentration of 2–3 × 10^5 cells/mL in RPMI 1640 supplemented with 10% FCS, streptomycin (100 μg/mL) and penicillin (100 Units/mL). The cells were kept in exponential growth at 37°C in 5% CO_2 humifified atmosphere by replenishing the cultures with fresh medium every 48–72 hr. Cell growth was determined at various time intervals by measuring the number of cells with a hemocytometer under a light microscope.

Animals

Adult albino Wistar rats (male, 200–300 g), used throughout this study, were bred in our animal house. Rats were injected i.p. either with saline (0.9% NaCl), ADR.HCl (10 mg/kg), hemin (10 mg/kg), or both ADR.HCl and hemin according to the protocol shown in Fig. 6. Rats were anesthetized with ethyl ether, samples of peripheral blood (2.5 mL) were removed, and the animals sacrificed by cervical dislocation.

DNA Probes

A full-length human liver cDNA clone, pCOX4.111 (700 bp), corresponding to subunit COX IV (pCOX41) [14], and a mitochondrial mt9 DNA fragment (846 bp), containing DNA sequences encoding $tRNA^{Ser}$, $tRNA^{Asp}$, and COXII (pHmt9) [15], were kindly donated by Dr. Eric Schon (Columbia University). Another cDNA fragment (451 bp) from rat pituitary tumor cells, corresponding to the gene COXII (pCOXII), was kindly donated by Dr. Priscilla Dannies (Yale University) [16].

DNA Isolation

Total and/or nuclear DNA was isolated according to the method of Davis *et al.* [17].

Morphological Examination of Cell Colonies

K-562 cells treated with ADR ± hemin or hemin alone were removed from culture, washed twice with drug-free fresh medium and subcultured in plasma clots, like MEL cells [18]. After 14 days, outgrown colonies were stained

with benzidine and hematoxylin and examined under a light microscope.

Detection of the DNA Apoptotic Ladder

Eight μg of each isolated DNA sample, prepared at different time intervals, was subjected to agarose (1.2%) gel electrophoresis in TBE buffer (0.089 M Tris-borate and 0.089 M boric acid, 0.02 M EDTA) at 8 V/cm. The DNA fragments were stained with ethidum bromide, visualized under UV light, and photographed with Polaroid™ 667 film.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated according to the method of Auffray and Rougeon [19]. Briefly, cells were washed twice with PBS, homogenized in 6M urea/3M LiCl solution and kept overnight at 0°C. The RNA was pelleted by centrifugation at 14,000 × *g* for 30 min. RNA was dissolved in 10 mM Tris.HCl/0.5% SDS and extracted with phenol/chloroform/isoamyl alcohol prior to ethanol precipitation. A constant amount of each RNA sample (25 μg) was electrophoretically separated on 1% agarose-2.2 M formaldehyde gel (3.4 V/cm for 12 hr), transferred onto a nylon membrane in 20 × SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) for 24 hr [20], and immobilized with UV light radiation for 3 min. Hybridization was performed according to the method of Church and Gilbert [21], using the ^{32}P-labeled DNA probes presented above. The filters were washed, exposed at low temperature (−70°C), and autoradiographed using Kodak™ XAR-5 film. Nylon membranes were stripped of the hybridized probe by immersion in sterile water at 100°C to be rehybridized once again.

Evaluation of Peripheral Blood Counts and Bone-Marrow Cellularity

Samples of peripheral blood, collected from control and drug-treated rats, were evaluated using a Coulter Counter, type S plus II, and WBC counts, as well as other peripheral blood counts, were recorded automatically. BMNCs were measured with a hemocytometer in aliquots of bone marrow aspirates removed from femurs. The latter were gently transferred under slightly hypotonic conditions that permitted lysis of RBC only, leaving the nucleated cells intact, as shown by light microscopy.

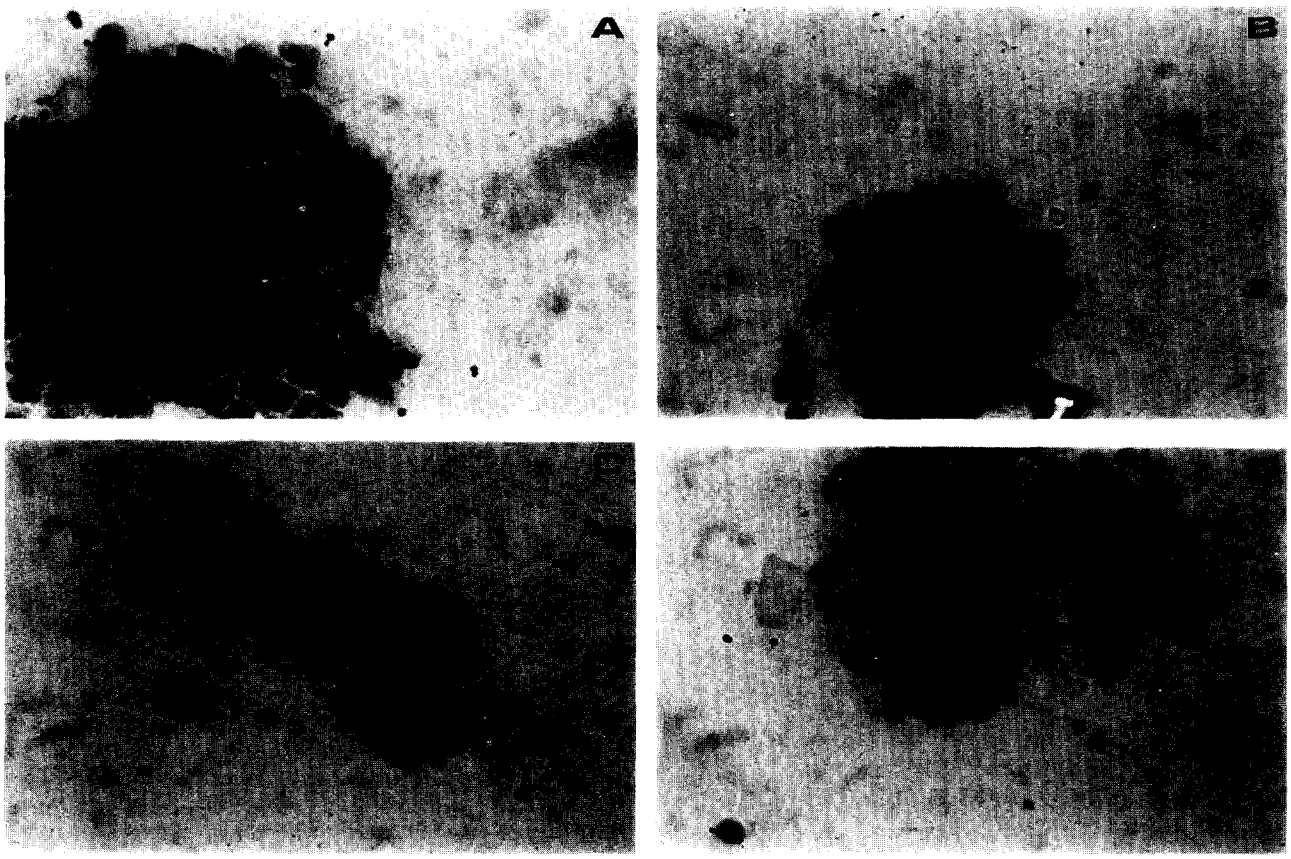

FIG. 1. The effects of co-treatment with hemin and ADR on morphology of K-562 cell colonies. K-562 cells were incubated with 30 μM hemin in the absence and presence of 3 × 10^{-8} M ADR. Six days later, cells were removed from culture, washed with fresh medium and subcloned in drug-free plasma clots. After 14 days, colonies were outgrown, stained with benzidine and hematoxylin, and examined using a microscope (× 400). Cells were incubated with no drug (A), hemin (B), ADR (C), and ADR + hemin (D).

Long-Term Bone-Marrow Cultures (LTBMCs)

Bone-marrow aspirates from control and drug-treated rats were prepared and evaluated for their ability to generate clusters and stroma as follows: bone marrow was flushed out from femurs removed from sacrificed rats with a sterile syringe equipped with a 21G needle filled with 10 mL McCoy's 5a medium [supplemented with 12% heat-inactivated FCS, 12% heat-inactivated horse serum, hydrocortisone (2.4×10^{-5} M), 0.75% MEM amino acid solution, 0.4% MEM nonessential amino acids, 1% sodium pyruvate, 0.6% sodium bicarbonate, 1% penicillin-streptomycin solution, 1% L-glutamine, 0.15% L-asparagine, 0.04% L-serine, and 0.4% MEM vitamins]. Dispersed bone-marrow cells filtered through gauze were placed in flasks (25 cm^2, Nunc, Dannish) and incubated at 33°C with the screw caps tightened. Cultures were then transferred for 2–3 hr each day in 5% CO_2 humidified atmosphere at 37°C, and cells fed once weekly by replacing half of the culture medium with an equal volume of fresh medium [22], thus maintaining LTBMCs viable for several weeks.

The ability of bone-marrow aspirates to develop a stromal cell microenvironment was evaluated arbitrarily, with 1+ indicating sporadic appearance of adherent stromal cells and 5+ indicating maximum coverage of the available surface on the bottom of the flasks by fibroblasts, reticular-type cells, endothelial cells, adipocytes, and macrophages.

Assessment of Clonogenic Potential of Bone-Marrow Cells in Plasma Clots

Samples of bone marrow removed from control and drug-treated rats (2×10^5 cells/400 μL in 4-well multidish Nunc, Dannish) were cultured in plasma clots containing 20% RPMI, 25% FCS, 1% bovine serum albumin, 1% asparagine, 1% beef embryo extract, 10^{-4} M β-mercaptoethanol, 0.9 units topaotatine, and 1% bovine citrate plasma, supplemented with 5% conditioned medium from the 5637 cell line, as defined previously [23–25]. Hemopoietic CFU-GM clusters containing 4 to 64 cells and fibroblastoid CFU-F clusters were scored 5 days later, because simultaneous growth of CFU-GM and CFU-F in the same dish could subsequently complicate the counting of clusters, and examined under an inverted light microscope.

RESULTS

ADR Induces Apoptotic Phenotype in K-562 Cells

As we have shown earlier, treatment of cells with ADR (3×10^{-8} M) killed the majority of K-562 cells [26]. However, a small portion of K-562 cells (<1%) that survived ADR treatment gave rise to small colonies in drug-free plasma clots. These colonies were composed of cells with a pycnotic nucleus and apoptotic morphology, as well as cells similar to control untreated cells. Apoptotic cells were markedly different from those seen in colonies developed from control (Fig. 1A) and/or hemin-treated cultures (Fig. 1B). The latter cells were smaller in size, synthesized hemoglobin as shown by staining with benzidine-H_2O_2 solution, but were not committed to terminal maturation, as expected [27]. Cotreatment of K-562 cells with hemin and ADR increased the proportion of replicating cells (~15%) and permitted them to survive and give birth to mixed colonies [26]. Some cells in the mixed colonies remained undifferentiated, others continued to produce hemoglobin, and still others exhibited apoptotic morphology similar to that of cells treated with ADR. These data suggest that treatment with ADR and subsequent culture led to accumulation of apoptotic cells. This finding implies that ADR promotes alteration(s) that are transmitted and expressed to their progenies later. Analysis of mixed colonies derived from hemin-ADR-treated cells suggests that hemin inhibits ADR to cause apoptosis in a portion of K-562 cells. These findings are consistent with our earlier observation that hemin selectively prevents, but does not abolish, ADR-induced cytotoxicity.

Hemin Counteracts ADR-Induced DNA Fragmentation in K-562-Cells

The data shown in Fig. 1 prompted us to investigate whether or not ADR, known to cause DNA degradation,

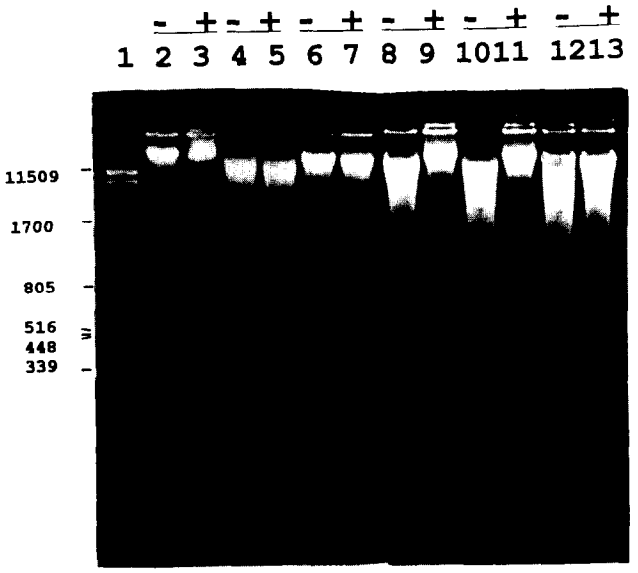

FIG. 2. Effects of hemin on dose-dependent DNA fragmentation in K-562 cells by ADR. K-562 cells were incubated for 48 hr at 37°C with various concentrations of ADR.HCl in the absence or presence of 30 μM hemin. At the end of incubation, nuclear DNA was isolated and subjected to agarose (1.2%) gel electrophoresis (8 μg of DNA per lane). (-) indicates incubation of cells in the absence of hemin and (+) in the presence of hemin. Lane 1: λDNA/Pst I digests served as molecular weight markers; DNA from control cells (lanes 2,3); and from cells treated with: 0.2 μM ADR.HCl (lanes 4,5), 0.5 μM ADR.HCl (lanes 6,7), 1 μM ADR.HCl (lanes 8,9), 2 μM ADR.HCl (lanes 10,11), and 5 μM ADR.HCl (lanes 12,13).

degrades cellular DNA by yielding an apoptotic ladder (DNA fragments of discrete size). Cells were exposed to varying concentrations of ADR for different times in the presence and absence of hemin (Fig. 2). DNA degradation with the characteristic pattern of internucleosomal ladder was observed in cells treated with concentrations of ADR larger than 1 μM. Treatment with 5 μM ADR caused more extensive degradation of DNA (rather than ladder-type damage). In the case of cotreatment of cells with ADR and hemin, ADR failed to cause apoptotic fragmentation of DNA. Only at the high concentration of 5 μM ADR was hemin unable to protect cells from DNA damage. The protective effect of hemin on the dose-dependent induction of DNA fragmentation was specific, because hemin failed to protect K-562 cells from MTX-induced DNA fragmentation (Fig. 3) under the same experimental conditions. Moreover, it was shown that ADR-induced DNA fragmentation in K-562 cells was also time-dependent (Fig. 4), because the apoptotic ladder of DNA was observed after 36 hr incubation of cells with 2 μM ADR at 37°C. Cotreatment with hemin, once again, protected cells from ADR-induced DNA damage at all times examined.

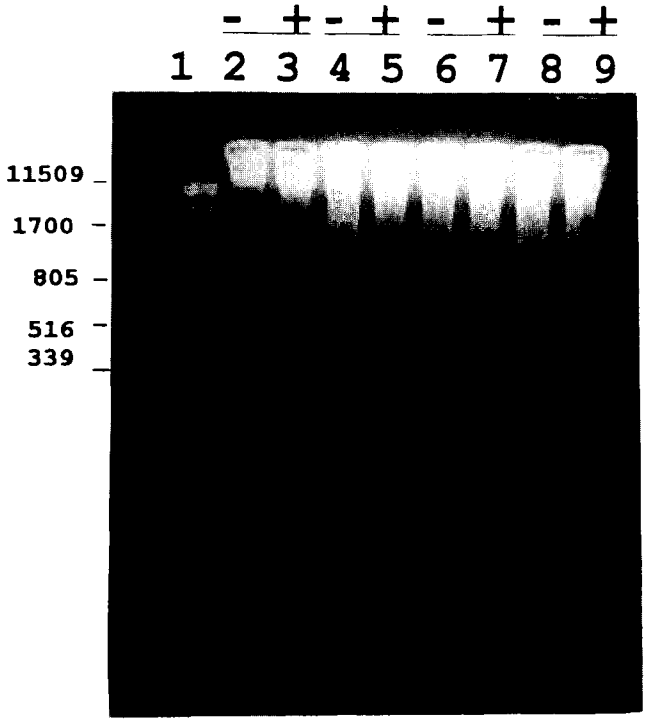

FIG. 3. Effects of hemin on dose-dependent DNA fragmentation in K-562 cells by MTX. K-562 cells were incubated for 48 hr at 37°C with various concentrations of MTX in the absence or presence of 30 μM hemin. At the end of incubation, total DNA was isolated and subjected to agarose (1.2%) gel electrophoresis (8 μg of DNA per lane). (-) indicates incubation of cells in the absence of hemin and (+) in the presence of hemin. Lane 1: λDNA/Pst I digests served as molecular weight markers; DNA from control cells (lanes 2,3); and from cells treated with: 0.5 μM MTX (lanes 4,5), 1 μM MTX (lanes 6,7), and 5 μM MTX (lanes 8,9).

ADR Causes Dose- and Time Dependent Repression of Two COX Genes

Our previous results showing that the mitochondrial COX enzyme is a target site for anthracyclines, such as ADR and DAU, and that inhibition of this enzyme activity is prevented by exogenously added hemin in a dose-dependent fashion prompted us to further explore the mechanism by which ADR inhibits this enzyme. As we mentioned in an earlier report [9], ADR may impair the biosynthesis of this enzyme at the transcriptional level in addition to inhibiting enzyme activity directly. The first of these possibilities appeared reasonable, given the frame of action of anthracyclines at the DNA level [2]. Two COX genes were chosen for this study. The first is mitochondrial and encodes the subunit COXII, and the other is nuclear and encodes the subunit COXIV. We assessed the steady-state levels of the mRNAs of these two genes with two ^{32}P-labeled DNA probes (pCOX41 and pHmt9). As indicated in Fig. 5, treatment of K-562 cells with varying concentrations (0.2–5.0 μM) of ADR suppressed expression of the COXIV and COXII genes. Exposure of ADR-treated cells to hemin did not reverse this suppression. As shown in Fig. 5B, a second band was visualized at the lower portion of the autoradiograph. This band corresponds to the $tRNA^{Ser}$ and $tRNA^{Asp}$ detected with the pHmt9 probe and was not detected when a similar membrane was hybridized with the pCOXII probe

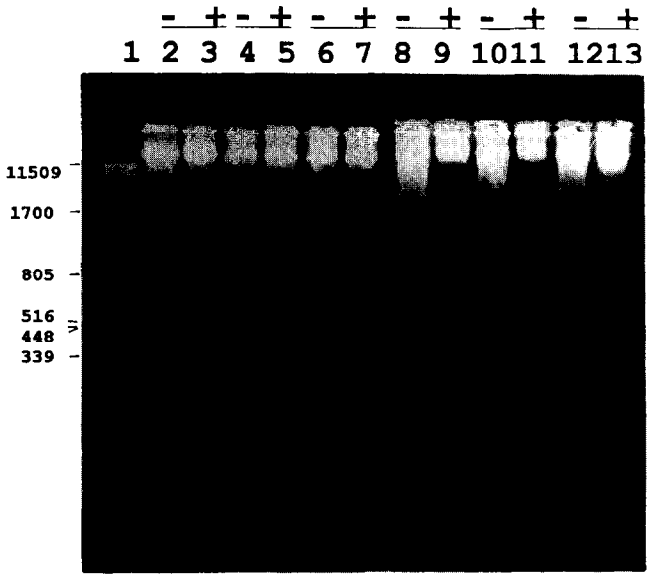

FIG. 4. Effects of hemin on time-dependent DNA fragmentation in K-562 cells by ADR. K-562 cells were incubated for different times at 37°C with 2 μM ADR.HCl in the absence or presence of 30 μM hemin. At the end of incubation nuclear DNA was isolated and subjected to agarose (1.2%) gel electrophoresis (8 μg of DNA per lane). (-) indicates incubation of cells in the absence of hemin and (+) in the presence of hemin. Lane 1: λDNA/Pst I digests served as molecular weight markers; DNA from control cells (lanes 2,3); and from cells incubated with ADR.HCl for 12 hr (lanes 4,5), 24 hr (lanes 6,7), 36 hr (lanes 8,9), 48 hr (lanes 10,11), and 72 hr (lanes 12,13).

containing only a piece of the COXII gene (data not shown).

ADR-Induced Bone-Marrow Cytotoxicity in Rats: the Effect of Hemin

Because hemin counteracts ADR-induced cytotoxicity *via* different mechanisms, we attempted to determine whether or not hemin exerts any beneficial effect on bone-marrow cellularity in ADR-myelosuppressed animals. To this end, we carried out a series of *in vivo* studies, presented here.

Preliminary studies from our laboratory have shown that a single injection of 10 mg/kg ADR.HCl led to myelosuppression in rats. ADR injected i.p. led to a reduction in peripheral WBCs 6 days following treatment, and administration of hemin alone, according to the protocol shown in Fig. 6, improved WBC counts above the control value (Fig. 6). Analysis of bone-marrow samples removed from ADR-treated rats revealed impaired bone-marrow functions and a lower number of BMNCs. In contrast to the stroma development and outgrowth of CFU-GM and CFU-F clusters seen in cultures of bone-marrow samples derived from control rats (Fig. 7), all bone-marrow samples derived from ADR-treated rats and plated in LTBMCs failed to develop stromal cell environment (0 to 2+) (Table I) and to give birth to CFU-GM and CFU-F clusters (Fig. 6). However, in one case (day 6), the number of CFU-GM clusters observed in a few rats was quite high. This could be attributed to the idiosyncracy of these animals. Injection of hemin alone also stimulated bone-marrow cell growth (Fig. 6) and preserved stromal cell environment (4+ to 5+) (Table I). However, the effect of hemin on colony formation was transient, because the number of CFU-F clusters increased by day 2, reached a higher level by day 6 and 9, and approached initial levels by day 12. Injection of hemin in ADR-treated rats did not substantially improve the number of BMNCs. Samples of bone-marrow cells derived from rats injected with both ADR and hemin developed higher numbers of CFU-GM and CFU-F clusters, compared to animals treated with ADR alone (Fig. 6). Similarly, bone-marrow samples derived from rats injected with both ADR and hemin developed adequate stromal cell environment (3+ to 4+) in LTBMCs, compared to rats injected with ADR alone (Table I).

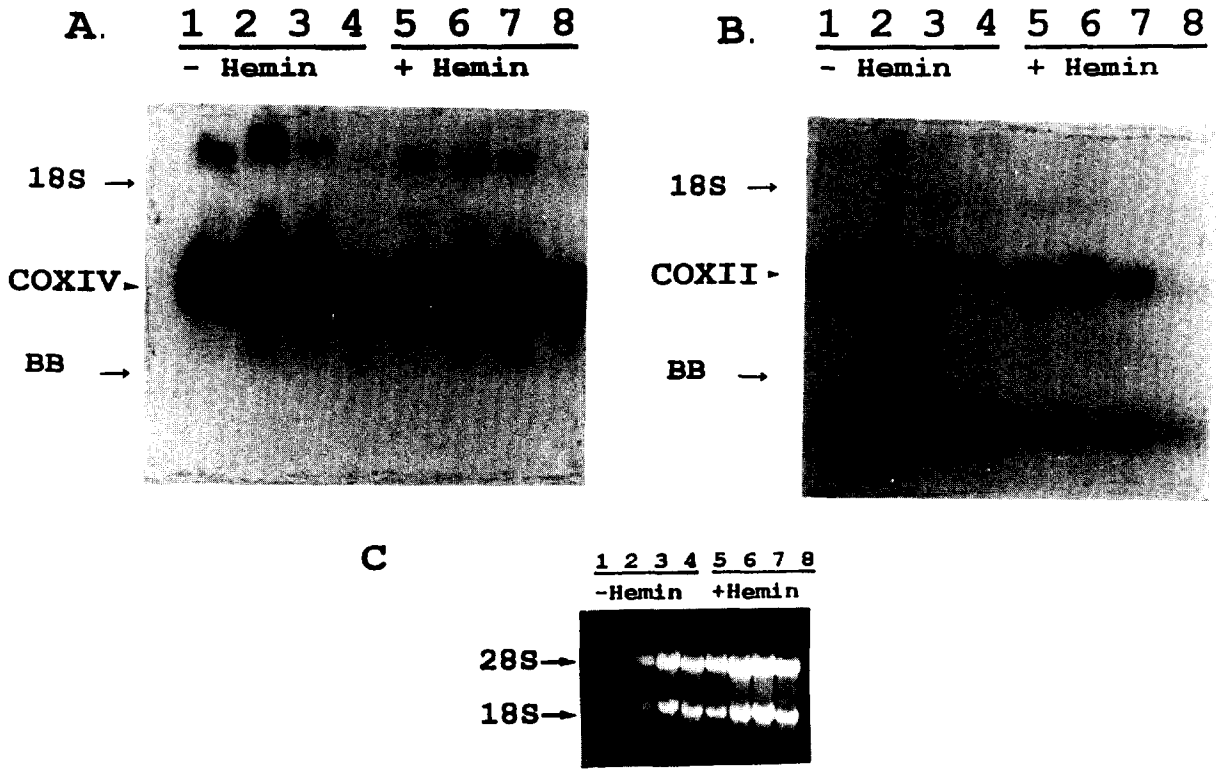

FIG. 5. ADR-induced dose-dependent effects on COX gene expression in K-562 cells in the absence or presence of hemin. K-562 cells were incubated for 48 hr at 37°C with various concentrations of ADR.HCl in the absence or presence of 30 μM hemin and total RNA was isolated. Samples (25 μg) of each RNA were electrophoretically separated on 1% agarose-2.2 M formaldehyde gel, transferred onto a nylon membrane, and hybridized at 65°C, first with ^{32}P-labelled DNA fragment (pCOX41) coding for nuclear COXIV mRNA (A), and then with a mitochondrial COXII DNA probe (pHmt9) (B). The membranes were washed at 65°C and autoradiographed. Panel C shows the corresponding ethidium bromide staining patterns of electrophoresed RNA samples (the positions of 28 S and 18 S rRNAs are indicated). RNA prepared from K-562 cells grown either without ADR.HCl (lanes 1,5) or with 0.2 μM ADR.HCl (lanes 2,6), 2μM ADR.HCl (lanes 3,7), and 5 μM ADR.HCl (lanes 4,8).

DISCUSSION

We investigated the mechanism(s) by which ADR and hemin interact in human K-562 cultured cells, as well as in rat bone-marrow cells, *in vivo*. In particular, we evaluated the effects of hemin on 3 related effects induced by ADR: (a) apoptosis; (b) inhibition of cytochrome c oxidase at the transcriptional level; and (c) myelosuppression *in vivo*. Analysis of these 3 effects could uncover how ADR exerts antineoplastic and myelosuppressive action (bone-marrow cytotoxicity) and demonstrate whether or not hemin was able to restore cell growth, DNA structural integrity, and cytochrome c oxidase activity in ADR-treated cells and bone marrow. From the results shown in Figs. 1–4, it is clear that ADR promotes apoptosis in cultured K-562 cells, as shown by the morphological appearance of K-562 cells and DNA fragmentation. It is particularly interesting that hemin, an agent that prevents ADR-induced cytotoxicity [9] and stimulates differentiation of K-562 cells [27], selectively reduces the frequency of apoptosis caused by ADR, but not by MTX, another antineoplastic agent. This selective action of hemin may be attributed to the fact that both hemin and ADR interact at the chromatin level [26], as well as at the level of mitochondrial COX (an enzyme pivotal for cell respiration) [9].

Northern blot hybridization analysis (Fig. 5) indicated that ADR (which inhibits COX activity [9] represses the expression of two COX genes, one mitochondrial and the other nuclear, although to a different extent. ADR alone suppressed the expression of both the mitochondrial COXII gene and the nuclear COXIV gene, and, in the presence of hemin, the mitochondrial COXII gene was more susceptible than the nuclear gene to the cytotoxic action of ADR. This ADR-induced suppression of COX genes could play a

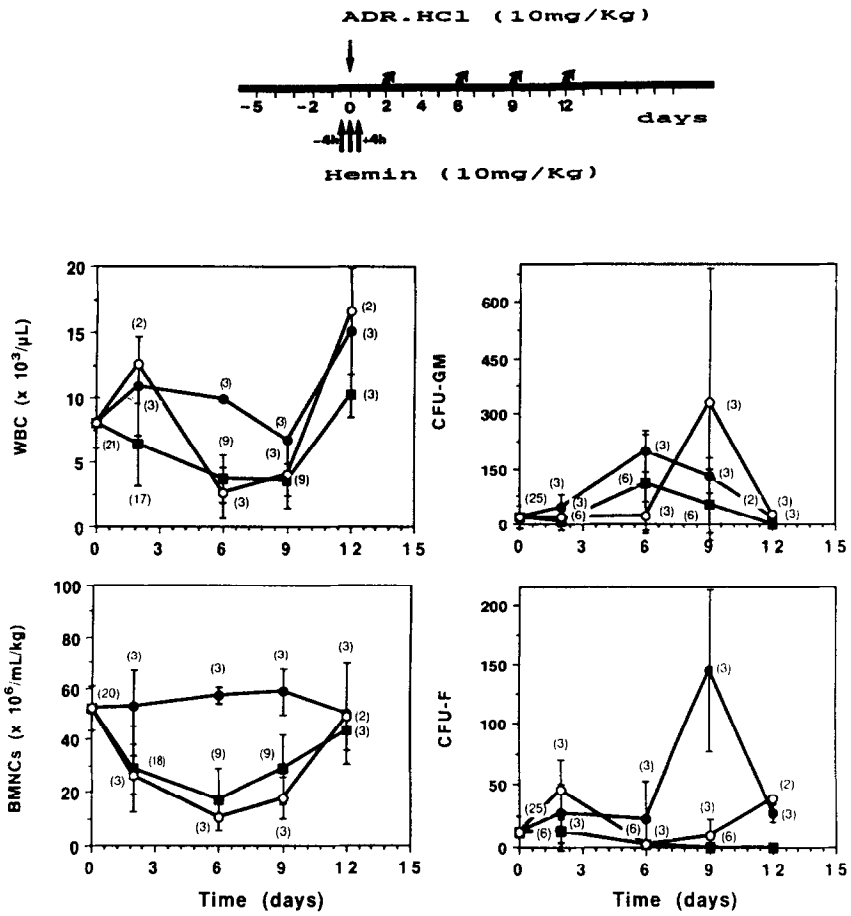

FIG. 6. Time-dependent effects of ADR and hemin on peripheral blood and bone marrow in i.p.-injected rats. Rats were injected i.p. either with 0.9% NaCl or ADR.HCl with or without hemin, as indicated in the upper panel. WBC counts and BMNCs were evaluated several days thereafter. Samples of bone marrow (2×10^5 cells/400 μL in 4-well multidish), removed at various times, were cultured in drug-free plasma clots, and the number of hemopoietic CFU-GM clusters and fibroblastoid CFU-F cells were counted microscopically 5 days later. Each point represents the mean value (±SD) of measurements obtained from (N) numbers of rats cited in parentheses. The value of y axis at time "0" represents the control value obtained from rats injected with 0.9% NaCl alone. Rats treated with: hemin alone (—●—), ADR.HCl alone (—■—) and both ADR.HCl and hemin (—○—). *Upper Panel:* Protocol of i.p. injection of ADR.HCl and hemin in rats. Vertical arrows indicate time-points for injection of ADR and hemin. Time-points for peripheral blood sampling and bone marrow removal are indicated by curved upwards arrows.

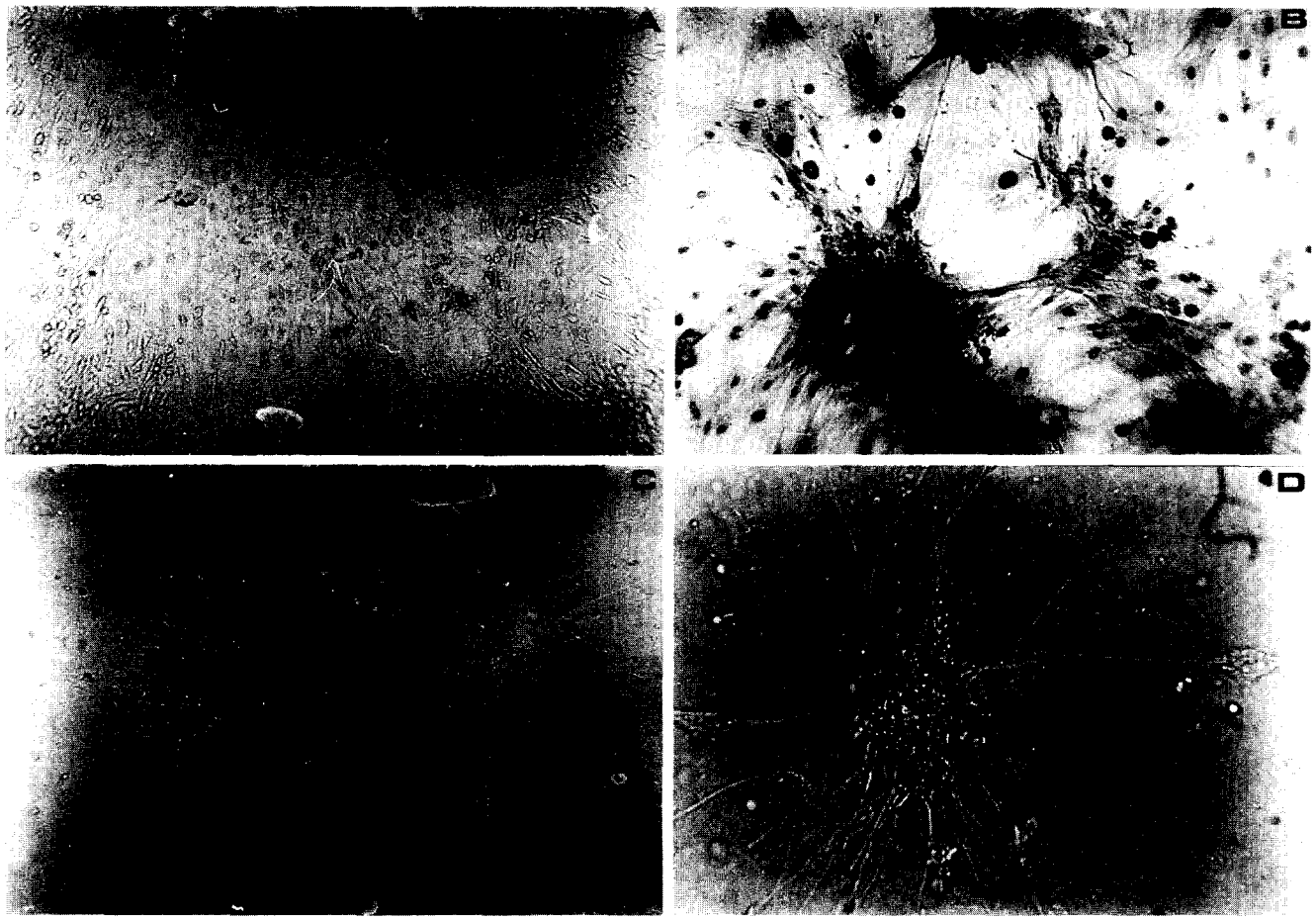

FIG. 7. Morphology of bone marrow stroma and CFU-GM and CFU-F clusters outgrown in cultures of bone marrow cells removed from untreated rats. (A) Well-developed (4+ to 5+) stromal cell environment in LTBMCs obtained from bone marrow samples of untreated control rats (May-Grunwald/Giemsa stain) (× 100). (B) Detailed morphology of well-developed (4+ to 5+) stromal cell environment in LTBMCs prepared from bone-marrow samples of untreated control rats (× 400). (C) CFU-GM clusters outgrown from rat bone-marrow cells plated in drug-free plasma clots (× 100) (see downward arrows). (D) CFU-F cluster outgrown from rat bone-marrow cells plated in drug-free plasma clots (× 400).

critical role in the biosynthesis of the corresponding subunits and the overall final assembly of the COX holoenzyme, leading to inhibition of COX enzyme activity overall.

Whatever the precise mechanism of hemin-ADR antagonism, hemin could offer a protective effect in myelosuppressed rats, because it prevented cytotoxicity induced by ADR [28] and apoptosis. If this were true, then the results could be of clinical value for ADR-treated patients. A single i.p. injection of ADR in rats caused reversible myelosuppression, characterized by a reduction in peripheral WBCs and failure of bone marrow to generate colonies and develop stromal cell environment (data from Figure 6 and Table I). It is well known that during hemopoiesis there is a constant renewal of blood cells from their hemopoietic progenitors *via* hemopoietic growth factors. ADR, which induces tumor cell death in K-562 cells, may also cause apoptosis in normal bone-marrow cells. Such a mechanism could explain the reduction of hemopoietic progenitors seen in ADR-treated animals, as shown in Fig. 6 by the reduction in the number of cells forming CFU-GM and CFU-F colonies, as well as by the reduction of counts observed in peripheral blood. Although it is difficult to distinguish which of the two major types of bone-marrow cell populations (i.e. hemopoietic or fibroblastoid) is preferentially more susceptible to ADR, the data presented indicate that stroma fibroblastoid cells were more susceptible to ADR than hemopoietic progenitors. Neither CFU-F clusters nor stroma were developed from bone-marrow samples derived from the majority of ADR-treated rats. Injection of hemin alone promoted development of the stromal cell environment and outgrowth of CFU-GM and CFU-F clusters. The clusters formed by bone-marrow samples removed from rats injected only with hemin after 6 and 9 days resulted from increased cell renewal of bone-marrow progenitor cells. This observation is in agreement with earlier reports claiming that hemin enhances the cell renewal capacity of erythroid CFU-Es, BFU-Es [29, 30, 31], as well as that of myeloid progenitors [32, 33]. Hemin may also act directly as a growth factor to control blood-cell production by inhibiting apoptosis [11] or indirectly by trig-

TABLE 1. Development of stromal cell environment in long-term bone-marrow cultures (LTBMCs)

Time of bone-marrow sampling (day)	Time of evaluation of LTBMCs (days)	Development of stromal cell environment		
		ADR.HCl	Hemin	ADR.HCl + hemin
2nd	6–9	damaged cells (3)	+++ (6)	damaged cells (3)
	10–15		++++ (5)	
	16–20		++++ (4)	
6th	6–9	+ (6)	++ (5)	ND
	10–15	+ (6)	+++ (5)	
	16–20	+ (6)	++++ (2)	
9th	6–9	+ (5)	+++ (6)	++ (2)
	10–15	++ (5)	++++ (5)	++++ (2)
	16–20	++ (5)	+++++ (2)	+++++ (1)
12th	6–9	damaged cells (3)	+++ (6)	++ (1)
	10–15		+++ (2)	+++ (1)
	16–20		++++ (2)	ND

Stromal cell environment in LTBMCs from control rats, injected with an equal volume of saline: (a) 6–9 days: +++ (16); (b) 10–15 days: +++ (15); (c) 16–20 days: ++++ (11). (Fewer samples of bone marrow were evaluated on certain occasions due to detachment of cells.) LTBMCs were evaluated for stromal cell environment as described in Materials and Methods. The number in parentheses indicates the number of bone-marrow samples evaluated in each case and the results are presented in arbitrary units. ND, not determined due to technical difficulties.

gering macrophages or other mononuclear cells to produce multilineage growth factors [33]. Injection of hemin in ADR-treated rats accelerated recovery from myelosuppression (as indicated by an increase in WBCs), preserved bone-marrow stromal cell environment, and stimulated outgrowth of CFU-GM and CFU-F clusters. It is possible that hemin exerts beneficial effects on bone marrow of ADR-myelosuppressed rats by promoting hemopoietic activity in the presence of ADR or by correcting hemopoietic abnormalities as observed in treatment with azidothymidine [32–34].

It is possible that hemin exerts a counteractive action on ADR-induced cytotoxicity by acting at various levels, interacting either directly or indirectly with ADR by preserving vital target sites, such as the mitochondrial COX enzyme in transformed K-562 cells and normal bone-marrow cells [9, 26, 28, 35, 36]. Alternatively, hemin and ADR can antagonize each other at the level of DNA by affecting its conformation and transcription. The latter possibility could explain the ability of hemin to prevent apoptosis. Whether or not apoptosis and inhibition of COX gene expression are related to each other and whether or not both these effects are responsible for antineoplastic activity and myelosuppression remains to be seen. Further investigations are needed to uncover the molecular events in the interaction between hemin and ADR to optimize their coadministration for the best possible protection of bone marrow during ADR therapy for nonhemopoietic malignancies.

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References

1. Chang J, Allen TD and Dexter TM, Long-term bone marrow cultures: Their use in autologous marrow transplantation. *Cancer Cells* **1:** 17–24, 1989.
2. Di Marco A, Adriamycin (NSC-123127): mode and mechanism of action. *Cancer Chemother Rep* [Part 3] **6:** 91–106, 1975.
3. Tritton TR and Yee G, The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science* **217:** 248–250, 1982.
4. Mimnaugh EG, Kennedy KA, Trush MA and Sinha BK, Adriamycin-enhanced mebrane lipid peroxidation in isolated rat nuclei. *Cancer Res* **45:** 3296–3304, 1985.
5. Doroshow JH, Effect of anthracycline antibiotics on oxygen radical formation in rat heart. *Cancer Res* **43:** 460–472, 1983.
6. Tewey KM, Rowe TC, Yang L, Halligan BD and Liu LF, Adriamycin induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* **226:** 466–468, 1984.
7. Bachmann E, Weber E and Zbinden G, Effects of seven anthracycline antibiotics on electrocardiogram and mitochondrial function of rat hearts. *Agents Actions* **5:** 383–393, 1975.
8. Muhammed H, Ramasarma T, Ramakrishna Kurup CK, Inhibition of mitochondrial oxidative phosphorylation by adriamycin. *Biochim Biophys Acta* **722:** 43–50, 1982.
9. Papadopoulou LC and Tsiftsoglou AS, Mitochondrial cytochrome c oxidase as a target site for daunomycin in K-562 cells and heart tissue. *Cancer Res* **53:** 1072–1078, 1993.
10. Ling YH, Priebe W and Perez-Soler R, Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. *Cancer Res* **53:** 1845–1852, 1993.
11. Thompson CB, Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456–1462, 1995.
12. Myers CD, Katz FE, Joshi G and Millar JL, A cell line secreting stimulating factors for CFU-GEMM culture. *Blood* **64:**152–155, 1984.
13. Lozzio CB and Lozzio BB, Human chronic myelogenous leukemia cell line with positive Philadelphia chromosome. *Blood* **45:** 321–334, 1975.
14. Zeviani M, Nakagawa M, Herbert J, Lomax MI, Grossman LI, Sherbany AA, Miranda AF, DiMauro S and Schon EA, Isolation of a cDNA clone encoding subunit IV of human cytochrome c oxidase. *Gene* **55:** 205–217, 1987.

15. Mita S, Schmidt B, Schon EA, DiMauro S and Bonilla E, Detection of "deleted" mitochondrial genomes in cytochrome-c oxidase-deficient muscle fibers of a patient with Kearns-Sayre syndrome. *Proc Natl Acad Sci USA* **86:** 9509–9513, 1989.
16. Van Itallie CM and Dannies PS, Estrogen induces accumulation of the mitochondrial ribonucleic acid for subunit II of cytochrome oxidase in pituitary tumor cells. *Mol Endocrin* **2:** 332–337, 1988.
17. Davis LG, Dibner MD and Battey JF, Preparation of DNA from eukaryotic cells: General method. In: *Basic Methods in Molecular Biology*, pp. 44–46. Elsevier Science Publishing Co., Inc., New York, 1986.
18. Gussela JF, Geller R, Clarke B, Weeks V, Housman D, Significance of the cell cycle in commitment of murine erythroleukemia cells: a stochastic analysis. *Cell* **9:** 221–229, 1976.
19. Auffray C and Rougeon F, Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem* **107:** 303–314, 1980.
20. Sambrook J, Fritsch EF, Maniatis T, Molecular Cloning, 2nd Ed, Cold Spring Harbor Laboratory Press, 1989.
21. Church GM and Gilbert W, Genomic sequencing. *Proc Natl Acad Sci* USA **81:** 1991–1995, 1984.
22. Dexter TM, Allen TD and Lajtha LG, Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J Cell Physiol* **91:** 335–344, 1977.
23. McLeod DL, Shreeve MM and Axelrad AA, Improved plasma culture system for production of erythrocytic colonies *in vitro*: Quantitative assay method for CFU-E. *Blood* **44:** 517–534, 1974.
24. Steinberg HN, Tsiftsoglou AS and Robinson SH, Loss of suppression of normal bone marrow colony formation by leukemic cell lines after differentiation is induced by chemical agents. *Blood* **65:** 100–106, 1985.
25. Piacibello W, Sanavio F, Severino A, Morelli S, Vaira AM, Stacchini A and Aglietta M, Opposite effect of Tumor Necrosis Factor α on granulocyte colony stimulating factor and granulocyte-macrophage colony-stimulating factor-dependent growth of normal and leukemic hemopoietic progenitors. *Cancer Res* **50:** 5065–5071, 1990.
26. Tsiftsoglou AS, Wong W and Robinson SH, Analysis of hemin-induced protection of human hemopoietic cells from the cytotoxic effects of anthracyclines. *Cancer Res* **48:** 3566–3570, 1988.
27. Tsiftsoglou AS, Wong W, Tsamadou AI and Robinson SH, Cooperative effects of hemin and anthracyclines in promoting terminal erythroid maturation in K562 human erythroleukemia cells. *Exp. Hematol.* **19:** 928–933, 1991.
28. Tsiftsoglou AS, Wong W, Wheeler C, Steinberg HN and Robinson SH, Prevention of anthracycline-induced cytotoxicity in hemopoietic cells by hemin. *Cancer Res* **46:** 3436–3440, 1986.
29. Monette FC and Holden SA, Hemin enhances the in vitro growth of primitive erythroid progenitor cells. *Blood* **60:** 527–530, 1982.
30. Monette FC, Holden SA, Sheehy MJ and Matzinger EA, Specificity of hemin action in vivo at early stages of hematopoietic cell differentiation. *Exp. Hematol* **12:** 782–787, 1984.
31. Kaye FJ, Weinberg RS, Schofield JM and Alter BP, The effect of hemin in vitro and in vivo on human erythroid progenitor cells. *Int J Cell Cloning* **4:** 432–446, 1986.
32. Chertkov JL, Jiang S, Lutton JD, Levere RD and Abraham NG, Hemin stimulation of hemopoiesis in murine long-term bone marrow culture. *Exp Hematol* **19:** 905–909, 1991.
33. Abraham NG, Heme regulation of hematopoietic stem cell growth and development. In: *Concise Reviews in Clinical and Experimental Hematology*, (Ed. Murphy MJ.Jr) pp. 357–373, AlphaMed Press, Dayton, OH, 1992.
34. Abraham NG, Cherkov JL, Staudinger R, Jiang S, Lutton JD, Argani I. Levere RD and Kappas A, Long-term bone marrow stromal and hemopoietic toxicity to AZT: Protective role of heme and IL-1. *Exp Hematol* **21:** 263–268, 1993.
35. Papadopoulou LC, Wheeler C and Tsiftsoglou AS, Possible role of mitochondrial components in adriamycin-induced cytotoxicity of human leukemia cells. *J Chemotherapy* [Suppl 4] **1:** 1151–1154, 1989.
36. Wheeler C, Robinson SH, Tsiftsoglou AS, Interactions of daunomycin (DN) with heme and hemoproteins: implications for the mode of action of anthracycline drugs. *Blood* [Suppl 1] **64:** 177a, 1984.