Prevention of Anthracycline-induced Cytotoxicity in Hemopoietic Cells by Hemin

Asterios S. Tsiftsoglou, Willie Wong, Catherine Wheeler, Howard N. Steinberg, and Stephen H. Robinson

Charles A. Dana Research Institute, Harvard-Thorndike Laboratory and Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts 02215

ABSTRACT

Anthracyclines such as Adriamycin (ADR) and daunomycin markedly inhibit cell growth in vitro and in vivo. These studies demonstrate that 30 μM hemin, which induces hemoglobin synthesis in human and murine erythroleukemia cells in culture, markedly decreases the cytotoxicity of ADR in a variety of hemopoietic cell lines (K562, HEL-1, MEL-745, HL-60, and HEL-RP37) and in erythroid burst-forming cells from normal human marrow. Hemin failed to protect four of the five nonhemopoietic cell lines tested, including MCF-7 breast adenocarcinoma cells, C-205 colon carcinoma cells, mouse 3T3 fibroblasts, and mouse kidney VERO cells. Hemin did protect human neuroblastoma IMP-32 cells from ADR cytotoxicity; however, this nonhemopoietic cell line undergoes dendrite formation in response to hemin induction. Cytofluorographic analysis of cellular ADR content and labeling studies with [3H]daunomycin demonstrated that hemin decreases the intracellular accumulation of these anthracyclines by more than 50% in K562 erythroleukemia cells. These studies indicate that small doses of hemin prevent intracellular accumulation of anthracyclines and thereby markedly reduce anthracycline toxicity to cells. Since this protective effect is observed preferentially with hemopoietic cells, it is possible that this finding could be exploited to protect the bone marrow from the cytotoxic action of anthracyclines during therapy for nonhemopoietic tumors.

INTRODUCTION

Hemin induces differentiation, as evidenced by increased hemoglobin production, in both murine (MEL) and human (K562) erythroleukemia cells in culture (1–9). This effect of hemin is reversible (4). It is not associated with terminal maturation and limitation of proliferative capacity, even in MEL cells in which a number of agents can induce commitment to terminal maturation (6). No agent thus far has been shown to induce terminal maturation in the highly malignant K562 cell line. In studies involving use of combinations of potential inducing agents including hemin and anthracyclines, which can induce differentiation in human HL-60 promyelocytic leukemia cells (10), we observed that low concentrations of hemin protect K562 cells from the cytotoxic effects of ADR (11). Experiments were therefore performed to study this protective effect of hemin on a variety of cells, both hemopoietic and nonhemopoietic in nature.

MATERIALS AND METHODS

Materials. Hemin was purchased from Eastman Kodak, Rochester, NY and dissolved in slightly alkaline solution as described earlier (6). There was no difference in the pH of cultures which did or did not contain hemin, presumably because of the buffering capacity of the medium. ADR and DAU were obtained from Sigma Chemical Co., St. Louis, MO. The anthracyclines aclacinomycin, mussetamycin, pyrrocellomycin; BFU-E, burst-forming cell, erythroid; CFU-E, colony-forming cell, erythroid.

RESULTS

Prevention by Hemin of ADR-induced Inhibition of K562 Cell Growth. As shown in Fig. 1, incubation with 5 x 10^-5, 2 x 10^-5, and 1 x 10^-7 M ADR alone caused 24, 58, and more than 80% inhibition of K562 cell growth respectively after 72 h of incubation. More extensive inhibition of cell growth was observed with longer incubation periods. Exposure of cells to the same concentrations of ADR in the presence of 30 μM hemin resulted in a much smaller degree of inhibition. Cells treated with hemin and 5 x 10^-5 M ADR underwent virtually no suppression of cell growth whereas cells treated with hemin and 2 x 10^-4 or 1 x 10^-7 M ADR grew to levels up to four-fold higher than cells treated with the same concentrations of ADR alone. Microscopic examination revealed preservation of normal morphol-
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Fig. 1. Effect of hemin (Hε) on growth of Adriamycin-treated K562 cells. Exponentially growing K-562 cells were incubated with varying concentrations of ADR in the presence or absence of 30 μM hemin. Cell number was determined after 72 h incubation by counting cells in a Coulter Counter Model ZBI, Coulter Electronics, FL. Points, average of duplicate measurements.

Fig. 2. Effects of hemin (Hε) on ADR-induced inhibition of cell growth of hemopoietic and nonhemopoietic cells. Cells (1–2 × 10⁵/ml) were incubated in the presence of no drug, ADR (3 × 10⁻⁴ m), hemin (30 μM), or both ADR and hemin. Cell number was measured at several intervals, from 0–140 h. For simplicity, values shown here are from the 72-h time points which are on the linear portion of the growth curve. Growth of BU-F-E in normal bone marrow cultures was assessed from the number of burst colonies that developed in the presence of erythropoietin (3.0 units/ml) (16). A, hemopoietic cells; B, nonhemopoietic cells. MEL-745, murine erythroleukemia, clone 745-PC4; K-562, human erythroleukemia; HEL-1, human erythroleukemia; U-937, human histiocytic lymphoma; HL-60, human promyeloctic leukemia; J77, mouse fibroblast; IMF-32, human neuroblastoma cells; Colo-205, human colon carcinoma cell line C-205; VERO, monkey kidney cells; MCF-7, human breast adenocarcinoma cells. Columns, average of duplicate measurements.

ology in K562 cells treated with both ADR and hemin in contrast to the disintegration of these cells observed after treatment with ADR alone. No morphological differentiation was observed in cells treated with both ADR and hemin; there was, however, increased accumulation of hemoglobin, as shown by the appearance of benzidine staining and by spectrophotometric analysis of hemoglobin content (19) after 6 days of incubation (control, 1.5; hemin, 90.0; ADR, 22; and hemin/ADR, 83.0 μg hemoglobin/10⁷ cells). A similar but less marked protective effect of hemin was observed with K562 cells treated with DAU or MARC as compared to ADR at similar molar concentrations (data not shown).

Preferential Protection of Hemopoietic Cells from ADR-induced Cytotoxicity by Hemin. All of the cells studied were susceptible to ADR cytotoxicity as measured by changes in cell number in liquid cultures or colony formation in plasma clot cultures (Fig. 2A), although only a small inhibitory effect was observed in VERO cells (Fig. 2B). A substantial protective effect of hemin was observed with all of the hemopoietic cells tested (K562, HL-60, HEL-1, U-937, MEL cells, and BU-F-E) and with the IMP-32 neuroblastoma cell line. Hemin afforded no protection to C-205 carcinoma cells, MCF-7 mammary adenocarcinoma cells, 3T3 mouse fibroblasts, and VERO cells.

Time-dependent "Rescue" of ADR-treated Cells by Hemin. As shown in Fig. 3, hemin is capable of rescuing cells from ADR cytotoxicity when the cells have been previously exposed to ADR for up to but no longer than 12 h. Cells which had been pretreated with ADR (3 × 10⁻⁴ m) for 0, 3, 6, or 12 h and then incubated with 30 μM hemin alone exhibited a 25–30% higher rate of growth than cells incubated in the absence of hemin after the removal of ADR (Fig. 3B). Similar data were obtained with K-562 cells which were exposed to daunomycin for the same periods and then incubated with or without hemin (data not shown).

Inhibition of Hemin of Intracellular Accumulation of Anthracyclines in K-562 Cells. As shown in Fig. 4, hemin decreased the intracellular accumulation of ADR, as indicated by a shift of ADR fluorescence intensity from right to left, with every concentration of ADR tested; thus, hemin decreases the ability of ADR either to enter K562 cells or to bind to intracellular components; alternatively it may enhance ADR efflux from these cells. Similar results were obtained for both DAU and MARC in K562 and MEL cells (data not shown). These findings were supported by the experiments shown in Fig. 5 which showed that hemin markedly inhibited the intracellular accu-

mulation of [³H]DAU (the only commercially available radioactive anthracycline) in K562 cells. To substantiate that the cellular [³H] radioactivity was still in the form of [³H]daunomycin, an extract of cells that had been incubated with [³H]daunomycin for 30 min was analyzed by thin-layer chromatography in a 16:6:1 chloroform:methanol:water solvent system. The [³H] spot extracted from the K562 cells was identical to that obtained with the original [³H]daunomycin.

DISCUSSION

Hemin has been found to exert a large number of functions in a variety of cells and in vitro biological systems. These include regulation of iron uptake and heme biosynthesis (14), activation of protein synthesis by increasing a cyclic AMP-independent protein kinase which phosphorylates the initiation factor eIF-2 (20), induction of globin mRNA and hemoglobin synthesis in erythroleukemia cells (7–9), and enhancement of the growth of BU-F-E and CFU-E erythroid progenitor cells (21). Hemin also has a differentiation-inducing effect in some nonhemopoietic cells; for example, it helps to induce adipocyte differentiation in 3T3 cells (22) and neurite outgrowth in mouse neuroblastoma cells (23). There is recent evidence that hemin regulates transcription of globin genes in human erythroleukemia K562 cells.
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Fig. 3. A, time-dependent hemin (He)-induced rescue of K-562 cells from ADR cytotoxicity. Exponentially growing K-562 cells were incubated with no drug, hemin (3 x 10^{-7} M), or ADR (3 x 10^{-8} M). At various times ADR was removed by washing the cells with phosphate-buffered saline 3 times and the cells were recultured in the presence of hemin alone (3 x 10^{-7} M). At times thereafter as shown on the abscissa, cell number was determined with the use of a Coulter Counter. Control (O), hemin treatment only (•), and cells which were switched from ADR to hemin alone after 3 (A), 6 (D), 12 (C), 24 (•), 48 (D), 72 (V), 96 (i), or 118 h (x). Points, average of duplicate measurements. B, effect of hemin on growth of K-562 cells pretreated with Adriamycin. K-562 cells were exposed to ADR (3 x 10^{-8} M) for 0, 3, 6, or 12 h, then removed from culture, washed 3 times with drug-free fresh medium, and reincubated in the presence or absence of 30 μM hemin. Cell number 72 h later was determined with the use of a Coulter Counter. Points, average of duplicate measurements.

(2, 5) and the iso-1-cytochrome c gene in the yeast Saccharomyces cerevisiae (24). These observations suggest that hemin has a physiological role in regulating cell differentiation, particularly in hemopoietic cells but also to some extent in some nonhemopoietic cells. Consistent with this conclusion is the recent observation that murine erythroleukemia cells contain membrane receptors for hemin (25). Our laboratory has added evidence (33) that hemin may interact with specific regions of nuclear DNA, perhaps in this manner enabling it to exert an effect on transcriptional processes.

Taking these observations into account, there are several ways in which hemin could suppress the cytotoxic effects of ADR on cell growth: (a) hemin could interact with specific sites on nuclear DNA, competing with uptake of ADR, which is known to intercalate with DNA, and thus decreasing total ADR uptake into the cell as shown in Figs. 4 and 5; (b) hemin may displace ADR from sites on the plasma membrane of the cell. There is evidence that ADR exerts some of its cytotoxic activity at the level of the cell membrane (26), and recent studies in MEL cells have demonstrated the presence of hemin receptors on the plasma membrane (25); (c) hemin may act as a free radical scavenger and prevent reactive species generated through the action of ADR from causing cell damage (27, 29, 31); (d) hemin may form a direct complex with ADR and thereby prevent this toxic agent from interacting with critical sites within the cell. In preliminary experiments we have found that ADR does form a complex with hemin, either free hemin or the heme moiety of the mitochondrial protein cytochrome c (28). Complex formation between ADR and hemin in the medium could of course prevent ADR from gaining access to cultured cells. However, two observations argue against this being a major mechanism by which hemin protects cells from ADR toxicity: (a) this mechanism should apply indiscriminately to all types of cells; however, hemin was unable to rescue several
susceptible to this differentiative effect of hemin; we did not
differentiation is relatively weak, and it is also possible that the
differentiation (22). However, the effect of hemin on adipocyte
which hemin has been reported to help induce adipocyte differ
hand, hemin did not exert a protective effect on 3T3 cells, in
that we have recently confirmed in our laboratory. On the other
exception, this protective effect of hemin has been observed
only with hemopoietic cells. Perhaps only hemopoietic cells, in
protecting only certain kinds of cells from ADR cytotoxicity.
What explains the fact that hemin preferentially protects
certain cell types from the toxic effects of ADR? With one
exception, this protective effect of hemin has been observed
only with hemopoietic cells. Perhaps only hemopoietic cells, in
which hemin seems clearly to have regulatory functions, have
receptors for this molecule either at the level of the cell mem-
brane or perhaps in areas of the genome. This might explain
why the IMP-32 neuroblastoma cell line is also protected by
hemin, since hemin has a differentiative effect on these cells
causing them to elaborate dendritic processes, a phenomenon
that we have recently confirmed in our laboratory. On the other
hand, hemin did not exert a protective effect on 3T3 cells, in
which hemin has been reported to help induce adipocyte differ-
entiation (22). However, the effect of hemin on adipocyte differ-
entiation is relatively weak, and it is also possible that the
3T3 cell line that was studied in the present experiments is not
susceptible to this differentiative effect of hemin; we did not
have the opportunity to determine whether hemin induces
adipocyte formation in this particular subline.

These studies have implications in two broad areas. Eluci-
dation of the mechanism by which hemin protects some but not
all cells from the cytotoxic action of drugs like ADR should
lead to a clearer understanding of the mechanisms by which
both hemin and ADR exert their differentiative and toxic
effects, respectively, on living cells. Indeed, our preliminary
experiments are providing evidence that ADR may interact
with the heme moiety of mitochondrial cytochromes (28); this
would lead to disruption of aerobic metabolism and decreased
ability to deal with oxidant species within the cell (29). Fur-
thermore, these observations conceivably could have clinical
application. One of the major forms of toxicity in patients
treated with ADR is bone marrow suppression. Since hemin
preferentially protects hemopoietic cells from ADR-induced
cytotoxicity, it is possible that hemin could be used to protect
bone marrow cells during anthracycline therapy for nonhem-
opoietic malignancies. Clearly, much additional work will have
to be done to demonstrate that this hemin protective effect has
any clinical utility.

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Fig. 5. Inhibition by hemin of intracellular accumulation of [3H]daunomycin
in K-562 cells. Exponentially growing K-562 cells (6·10⁵ cells/ml) were
incubated with [3H]daunomycin (0.5 μCi/ml; 5 × 10⁻⁷ M) in the absence (○) or
presence (●) of hemin (30 μM). At the times indicated, aliquots of the cell
suspension were removed and the amount of radioactivity in the cells was
measured. Cellular radioactivity was identified specifically as [3H]daunomycin by
thin-layer chromatography (see text). Values, mean ± SE (bars) of 3-4 determi-
inations.
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