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Selective cancer cell cytotoxicity from exposure to dihydroartemisinin and holotransferrin

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Abstract

Rapid cell death, as evidenced by a decrease in cell counts, was observed when molt-4-lymphoblastoid cells, a human leukemia cell line, were exposed to holotransferrin (12 μM) and dihydroartemisinin (1–200 μM). Incubation with either compound alone was significantly less effective. Significantly less cell death was observed when normal human lymphocytes were exposed to a combination of these 2 drugs. Probit analysis of dose-response functions shows that the drug combination is approximately 100 times more effective on molt-4 cells than lymphocytes (LD_{50}s for molt-4 and lymphocytes were 2.59 μM and 230 μM, respectively). This drug combination may provide a novel approach for cancer treatment.

Keywords: Molt-4-lymphoblastoid cells; Lymphocytes; Holotransferrin; Dihydroartemisinin

1. Introduction

Most cancer cells express higher cell surface concentration of transferrin receptors than normal cells [1] and have high rates of iron influx via transferrin receptors. For example, the entire population of transferrin receptors on a mouse teratocarcinoma stem cell can be internalized within 6 min [2].

Artemisinin is a sesquiterpene lactone isolated from the plant *Artemisia annua* L. It is presently used in various countries as an antimalarial drug and has a potent effect on choloroquine-resistant malarial parasites [3]. An artemisinin molecule contains an endoperoxide bridge that can be induced by iron to form free radicals. Its antimalarial action is due to its reaction with the iron in free heme molecules in the malarial parasite with the generation of free radicals leading to cellular destruction [4].

The present study takes advantage of this property of artemisinin and targets it towards cancer cells with holotransferrin (i.e., iron-loaded transferrin). We found that in a human leukemia cell

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culture, combined exposure to holotransferrin and dihydroartemisinin, a more water-soluble active metabolite of artemisinin, can cause rapid destruction of cancer cells, whereas similar treatment had significantly less effect on normal human lymphocytes.

2. Materials and methods

Molt-4-lymphoblastoid cells and human lymphocytes were used in the experiment. Molt-4-lymph cells were purchased from the American Type Culture Collection (Rockville, MD). They are acute lymphoblastic leukemia cells from human peripheral blood. Cultures were maintained in RPMI-1640 (Gibco, Long Island, NY) supplemented with 10% fetal bovine serum (Hyclone, New Haven, CT). Cells were cultured at 37°C in 5% CO_2/95% air and 100% humidity, and were split 1:2 at a concentration of approximately 1×10^6/ml. Approximate cell numbers before the experiment were between 150×10^3–300×10^3 per ml. Human lymphocytes were isolated from fresh blood obtained from a healthy donor using a modification of the Ficoll-hypaque centrifugation method of Boyum [5]. In this method, 20–100 μl of whole blood obtained from a finger prick were mixed with 0.5 ml of ice-cold RPMI-1640 without phenol red (GIBCO, NY) in a 1.5-ml heparinized microfuge tube (Kew Scientific Inc., Columbus, OH). Using a Pipetman, 100 μl of cold lymphocyte separation medium (LSM) were layered at the bottom of the tube. The samples were centrifuged at 3500 rev./min for 2 min in a microfuge (Sorvall, Microspin model 245) at room temperature. The lymphocytes in the upper portion of the Ficoll layer were pipetted out. Cells were washed twice in 0.5 ml of RPMI-1640 by centrifugation for 2 min at 3500 rev./min in the microfuge. The final pellet consisting of approximately $0.4–2.0 \times 10^5$ lymphocytes, was resuspended in RPMI-1640. Cell viability was determined before experiments using trypan blue exclusion and found to be > 95%.

Cells (molt-4 and normal lymphocytes) were aliquoted in 0.1 ml volumes into microfuge tubes. Human holotransferrin (Sigma Chemicals, St. Louis, MO) was added to samples of the cells. Different concentrations of freshly-prepared dihydroartemisinin dissolved in complete medium were added 1 h later to the tubes. The final concentration of holotransferrin was 12 μM and dihydroartemisinin was either 1, 10, 50, or 200 μM. An equal volume of medium was added to control samples (i.e. samples without holotransferrin nor dihydroartemisinin). Cells were kept in an incubator at 37°C under 5% CO_2 and 95% air during the experiment. At 1, 2, 4, and 8 h after the addition of dihydroartemisinin, cell number was counted from a 10-μl aliquot from the samples using a hemocytometer. Cells were thoroughly mixed by repeated pipeting before an aliquot was taken for counting. In the case of molt-4 cells, cell viability was not determined because it is not correlated with cell loss as rapid cell disintegration was observed.

Data are expressed as percentage of cell count at a certain time-point compared to cell count at the time when dihydroartemisinin was added (time zero in Figs. 1 and 2). Time-response curves were compared by the method of Krauth [6]. The level of the curves, i.e. a_0 of the orthogonal polynomial coefficient, were compared with the median test. χ^2 was calculated with Yates's correction for continuity. Difference between 2 data points was also compared by the median test. A difference of $P < 0.05$ was considered statistically significant. The Probit analysis was used to determine LD_{50}s, i.e. the concentration of dihydroartemisinin that causes a decrease in cell count by 50% in 8 h, from the dose-response data.

3. Results

Percent cell count at different times after the addition of 200 μM of dihydroartemisinin of molt-4 cells and normal lymphocytes are shown in Figs. 1 and 2, respectively. In both molt-4 and lymphocytes, no significant difference in cell counts was observed between control samples and samples exposed to holotransferrin (12 μM) alone ($\chi^2 = 0.5$, df = 1, non-significant) during the 8-h incubation period. Compared to controls, a significant decrease in cell count (Fig. 1) was observed in molt-4 cells exposed to dihydroartemisinin alone ($\chi^2 = 4.5$, df = 1, $P < 0.035$ compared to

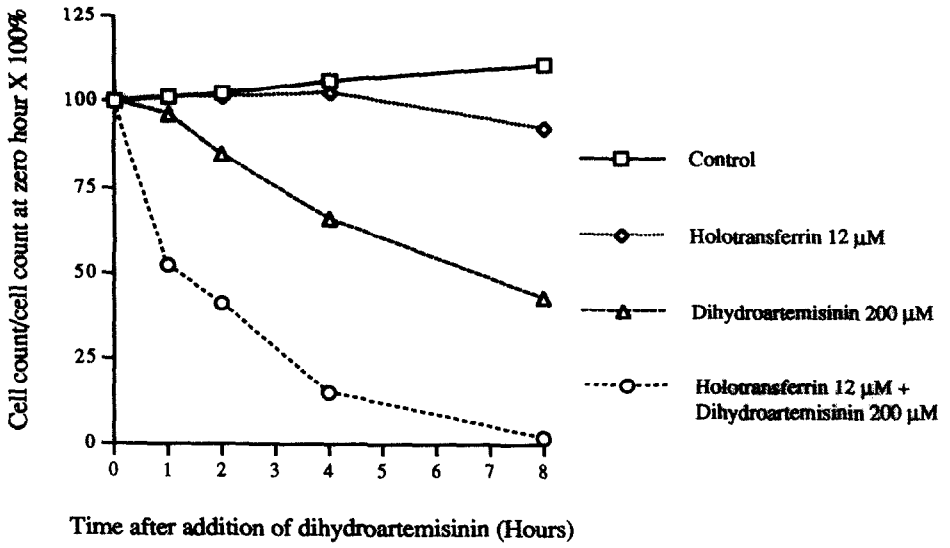

Fig. 1. Time-response curve of molt-4 cells incubated in holotransferrin (12 μM) and dihydroartemisinin (200 μM). Dihydroartemisinin was added at time zero, 1 h after the addition of holotransferrin. Each response curve is the average from 4 experiments.

control), and a combination of dihydroartemisinin and holotransferrin ($\chi^2 = 4.5$, df = 1, $P < 0.035$ compared to control). In addition, percent cell counts of the combined drug treatment were significantly less than those treated with dihyroartemisinin alone ($\chi^2 = 4.5$, df = 1, $P < 0.035$ compared to control).

In the case of lymphocytes, upon exposure to

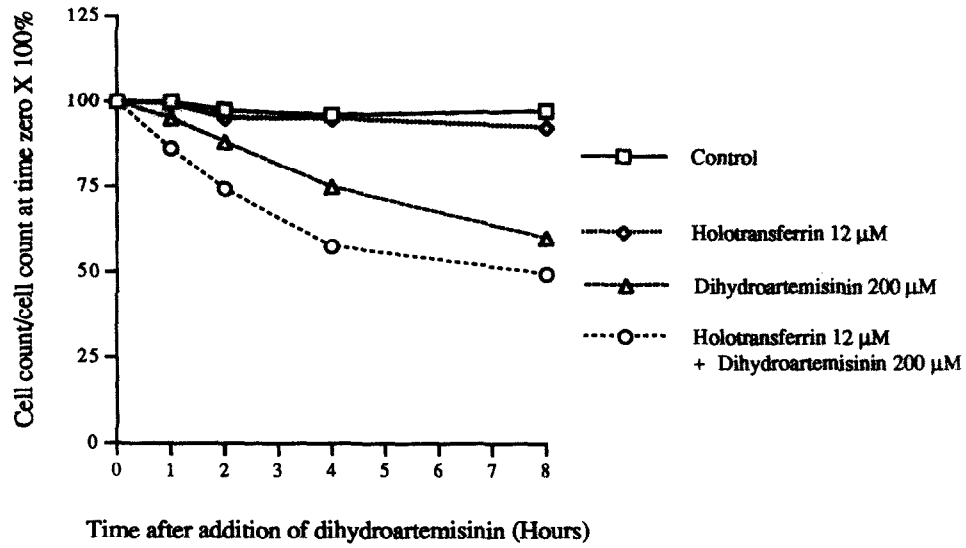

Fig. 2. Time-response curve of lymphocytes incubated in holotransferrin (12 μM) and dihydroartemisinin (200 μM). Dihydroartemisinin was added at time zero, 1 h after the addition of holotransferrin. Each response curve is the average from 4 experiments.

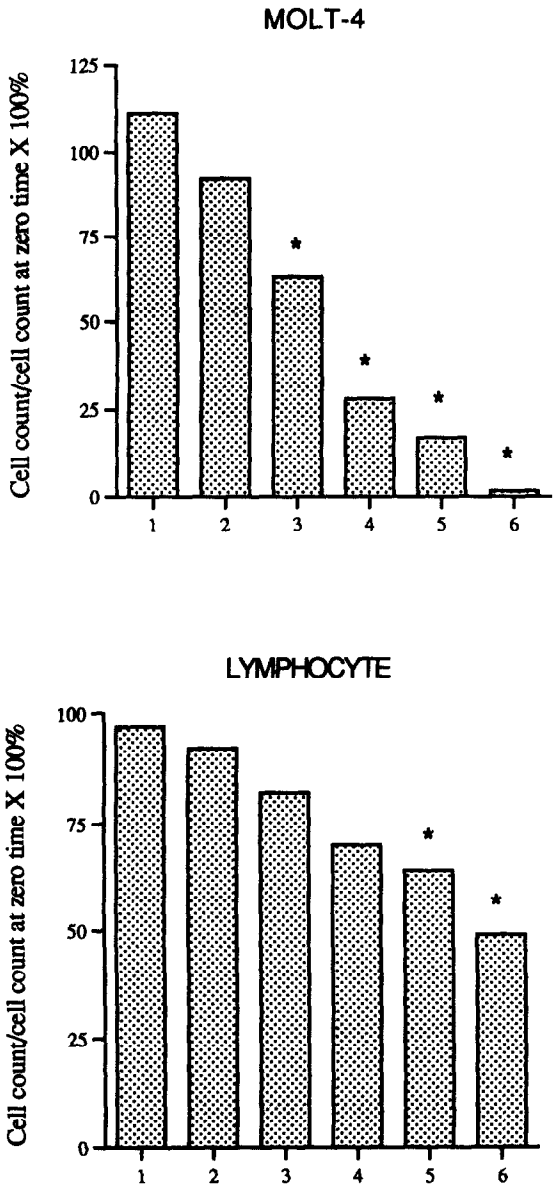

Fig. 3. Dose-response relationship of molt-4 cells (upper graph) and lymphocytes (lower graph) exposed to holotransferrin and dihydroartemisinin. Treatment 1 is control samples with no drug added. Samples in treatments 2–6 contained 0, 1, 10, 50, and 200 μM of dihydroartemisinin, respectively, plus 12 μM of holotransferrin. Holotransferrin was added at 1 h before the addition of dihydroartemisinin. Cell counts were done at 8 h after addition of dihydroartemisinin. Each bar represents the average from 4 experiments. * designates significantly different from control samples (treatment 1) at $P < 0.035$.

dihydroartemisinin alone or dihydroartemisinin plus holotransferrin, cell counts were significantly less than those of the controls ($\chi^2 = 4.5$, df = 1, $P < 0.035$ compared to control). However, the addition of holotransferrin did not significantly further enhance the effect of dihydroartemisinin alone ($\chi^2 = 0.5$, df = 1, no significant difference between the dihydroartemisinin alone and holotransferrin plus dihydroartemisinin response curves).

Fig. 3 shows the dose-response relationship to dihydroartemisinin of molt-4 and lymphocytes incubated in 12 μM of holotransferrin. Percent cell counts from samples at 8 h after addition of various concentrations of dihydrotransferrin were presented. Dose-dependent decreases in cell counts were observed. For molt-4 cells, a significant difference (from control samples, $\chi^2 = 4.5$, df = 1, $P < 0.035$) was observed at 1 μM of dihydroartemisinin and higher. Smaller decreases in cell counts were observed with lymphocytes under similar treatment conditions. A significant difference from control samples was observed only at a concentration of dihydroartemisinin of 50 μM and higher. Probit analysis of the data showed that the LD_{50}s for molt-4 cells and lymphocytes were 2.59 and 230 μM, respectively.

4. Discussion

Our data show that combined incubation in holotransferrin and dihydroartemisinin can selectively destroy human cancer cells, whereas the effect is significantly less on normal lymphocytes. Artemisinin alone has been shown to be toxic for cancer cells in vitro at 20–180 μM range [7]. The effect was found to be greater for hepatoma and embryonic lung cells than against human gastric cancer cells. However, serum concentrations at these levels cannot be reached in vivo. Addition of holotransferrin increases the potency and selectivity of the drug and may decrease the time of cell killing. In the combined treatment, considerable cell death was observed at a concentration of dihydroartemisinin of 1 μM after 8 h of incubation. Furthermore, there is reason to believe that artemisinin can work at lower concentrations in vivo than in vitro. Culture medium may contain

19–30 μM of free iron and could cause destruction of dihydroartemisinin molecules before they can gain entry into cells.

This procedure will be most effective for the treatment of aggressive cancer, in which large numbers of transferrin receptors are expressed on the cell surface. However, the procedure may not be effective in the treatment of certain types of cancer. For example, some adult T-cell leukemias have defective internalization of transferrin receptors [8] and may not be susceptible to this treatment.

Artemisinin is a relatively safe drug with little side-effects even at high doses [3,9,10]. Oral doses of 70 mg/kg/day for 6 days have been used in humans for malaria treatment. Furthermore, more potent analogs of this and similar compounds are also available [11–14]. Higher efficacy of artemisinin action also can be achieved by other means. For example, artemisinin is more reactive with heme than free iron [15]. Heme can be introduced into cells using transferrin [16] or the heme-carrying compound hemoplexin [17,18]. The effectiveness of artemisinin also can be enhanced by increasing oxygen tension, decreasing intake of antioxidants, and blockade of peroxidase and catalase by drugs such as miconazole [4,19,20].

Our data are preliminary. In future research, the parameters of treatment, such as drug dosage and schedule of drug exposure can be explored to attain higher selectivity of destruction of cancer cells with relatively minimal effect on normal cells. In addition, different types of cancer cells should be studied to test the generality of the effect.

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