Abstract. Background: Artemisinin is a chemical compound extracted from the wormwood plant, Artemisia annua L. It has been shown to selectively kill cancer cells in vitro and retard the growth of implanted fibrosarcoma tumors in rats. In the present research, we investigated its mechanism of cytotoxicity to cancer cells. Materials and Methods: Molt-4 cells, in complete RPMI-1640 medium, were first incubated with 12 µM of human holotransferrin at 37°C in a humid atmosphere of 5% CO2 for one hour. This enhanced the iron supply to the cells. The cells were then pelleted and transferred to a complete RPMI-1640 containing 200 µM of an analog dihydroartemisinin (DHA) and incubation was started (0 h). In addition, some culture samples were treated with holotransferrin alone and some (controls) were assayed without neither holotransferrin nor DHA treatment. Cells were counted and DNA diffusion assay was used to evaluate apoptosis and necrosis in each sample at 0 h and at 1, 2, 4 and 8 h of incubation. Results: DHA treatment significantly decreased cell counts and increased the proportion of apoptosis in cancer cells compared to controls ($\chi^2=4.5$, df =1, $p<0.035$). Addition of holotransferrin significantly further decreased cell counts ($\chi^2=4.5$, df =1, $p<0.035$) and increased apoptosis ($\chi^2=4.5$, df =1, $p<0.035$). No necrotic cells were observed. Conclusion: This rapid induction of apoptosis in cancer cells after treatment with DHA indicates that artemisinin and its analogs may be inexpensive and effective cancer agents.

Artemisinin, a known anti-malarial drug (1-4), extracted from the wormwood Artemisia annua L, is a sesquiterpene lactone peroxide containing an endoperoxide moiety, which forms free radicals when it reacts with iron. Iron is necessary for cell division and proliferation. Compared to normal cells, cancer cells have a tumor aggressiveness-dependent higher number of cell surface transferrin receptors (5-6) that pick up iron via interaction with the plasma iron-carrying protein transferrin. Thus, cancer cells would be selectively more susceptible to the cytotoxicity of artemisinin because of their higher rates of uptake of iron. Our previous work, showing the anticancer efficacy of artemisinin in cell cultures, rats and humans (7-10) and the work of other investigators (11-12), is based on this assumption. Artemisinin and its analogs are relatively inexpensive and readily available in both natural and synthetic forms. Based on our encouraging in vitro and in vivo results (7-9), including humans (10), we envision artemisinin as a very effective drug for the treatment of cancer.

The aim of the present study was to explore the mechanism of the cytotoxicity of an analog of artemisinin, dihydroartemisinin in Molt 4 cells, a human lymphoblastoid leukemia cell line. For this purpose, we used the DNA diffusion assay (13-15). This assay is simple, sensitive and can be used to estimate apoptosis and necrosis. The assay is based on the principle that apoptotic cells have numerous alkali-labile sites (13) and that these sites, once exposed to alkaline conditions, yield small pieces of DNA. These pieces can readily diffuse in an agarose-matrix, giving the appearance of a hazy halo around the cell. This unique pattern of a DNA gradient diffused in agarose is characteristic of apoptotic cells and is readily distinguishable from necrotic cells (13). This assay involves mixing cells with agarose and making a microgel on a microscopic slide, then lysing the embedded cells with salt and detergents and treating them with an alkaline solution (pH>13). This causes breakage of DNA at abundant alkali-labile sites found in apoptotic cells and allows diffusion of small molecular weight DNA fragments in agarose. The cells are stained with the fluorescent dye YOYO-1 and the DNA diffusion pattern visualized under a fluorescent microscope. This allows the measurement of the number (percentage) of apoptotic cells in a population of cells.

Materials and Methods

Materials. All chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA), unless mentioned otherwise. Molt-4 cells and fetal bovine serum were purchased from ATCC (Rockville, MD, USA). RPMI-1640 culture medium was purchased
Dihydroartemisinin (DHA) was purchased from Calbiochem (Amresco, Solon, OH, USA) which artemisinin kills cancer cells, we used DHA, a more water soluble and more potent analog of artemisinin in cell culture medium at 37°C in a humid atmosphere of 5% CO2 for 1640 medium, were incubated with 12 μM of human holotransferrin in RPMI-1640 medium freshly supplemented with 10% fetal bovine serum and antibiotics. Cultures were divided 1:2 every 48 h to an approximate cell density of 0.5 million cells/ml and used for experiments after 24 h.

**Treatment of Molt-4 cells with dihydroartemisinin.** Samples of Molt-4 cells, at an approximate density of 1 million cells/ml in complete RPMI 1640 medium, were incubated with 12 μM of human holotransferrin in culture medium at 37°C in a humid atmosphere of 5% CO2 for one h. At the end of an hour, cells were pelleted and transferred to complete RPMI 1640 medium having 200 μM of DHA and incubation was started (0 h). In addition, some culture samples were treated with holotransferrin alone and some (controls) were assayed without holotransferrin and DHA treatments. Cell count and apoptosis were determined in each sample at 0 h and at 1, 2, 4, and 8 h of incubation. To minimize damage to DHA and cells, minimum indirect light was maintained during these experiments.

**Cell death and cell counts.** We used the DNA diffusion assay for estimation of cell death, apoptosis and necrosis. As dihydroartemisinin causes rapid cell death and elimination of dead cells, we also counted the cell number using a hemocytometer. Only normal cells were counted.

**DNA diffusion assay.**

Making agarose. This methodology has been described in detail elsewhere (13-15). Briefly, 70 mg of high resolution agarose 3:1 (Amresco, Solon, OH, USA) was boiled in 9 ml of distilled water in a microwave oven and 1 ml of 10X modified PBS (for one liter: 80 g NaCl, 2 g KCl, 2 g KH2PO4, 11.5 g anhydrous Na2HPO4 or 29 g Na3HPO4•7H2O, 32 gm tris hydrochloride, pH 7.4). After adjusting the volume to 10 ml by adding distilled water, the solution was boiled once more. The volume was again adjusted to 10 ml by adding distilled water and the solution was well mixed to provide a concentration of 0.7%. The agarose was then dispensed in small aliquots and maintained at 50°C for 24 h prior to use. Similarly, a 2% SFR agarose (Amresco, Solon, OH, USA) was prepared in PBS. Two hundred mg of agarose was suspended in 10 ml 1 x PBS and boiled in a beaker. As the agarose has a tendency to stick to glass, it requires gentle stirring during boiling. Loss of water during boiling was compensated for by adding an appropriate amount of distilled water at the end.

Preparing slides: MGE slides (Erie Scientific Co, Portsmouth, NH, USA) were coated with 50 μl of 0.7% agarose 3:1(first layer). Agarose was pipetted on the top-frosted part of the slide while holding the slide horizontally in the left hand between the thumb and index finger and smearing the agarose in one motion using a pipette tip held horizontally by the right hand. The slides were then air-dried. Cells from one ml of each sample were centrifuged and the pellet was suspended in 10 μl of PBS and mixed well with 50 μl of 0.7% agarose 3:1 for each slide. Fifty μl of this mixture was layered onto the precoated (with first layer of microgel) slides and a cover glass (24 x 50 mm2, Corning Glass Works, Corning, NY, USA) was placed over it to make a second layer of microgel. The slides were put in a cold steel tray kept on ice. After removing the cover glass, a third layer of 200 μl of 2% SFR agarose was layered on top of the second layer. The use of 2% SFR agarose is essential to control too much diffusion of DNA from apoptotic cells in agarose. After keeping slides for 2 min on ice, the cover glasses were removed and the slides were immersed and maintained for 10 min in a freshly made and cold lysing solution (1.25 M NaCl, 1 mM tetrasodium EDTA, 5 mM Tris, 0.01% sodium lauroyl sarcosine, 0.2% DMSO and 300 mM NaOH).

Neutralization and staining of microgels: Slides for neutralization were immersed in freshly prepared 20 mM Tris, pH 7.4 in 50% ethanol with 1mg/ml of spermine for 15 min. This step was repeated once more. The slides were air-dried. One slide at a time was stained with 50 ml 0.25µM YOYO-1 in 2.5 % DMSO and 0.5% succrose.

The percentage of apoptotic cells with diffuse DNA and a hazy outline were calculated from a total of 1000 cells analyzed (normal plus apoptotic).

**Data analysis.** The percent cell count and percent of cells showing apoptosis from samples treated and not treated with holotransferrin at different time points after the addition of DHA were plotted. Response curves were compared using the method of Krauth (16).

**Results**

The cell counts and percent of apoptosis are shown in Figures 1 and 2, respectively. DHA treatment significantly decreased the cell counts and increased apoptosis in cancer cells compared to controls ($\chi^2=4.5, df=1, p<0.035$). The addition of holotransferrin further significantly enhanced the DHA-induced decrease in cell count ($\chi^2=4.5, df=1, p<0.035$) and increase in percentage of cells undergoing apoptosis ($\chi^2=4.5, df=1, p<0.035$). There was a significant difference in changes in cell counts and apoptosis scores between holotransferrin-alone treatment and controls. In both cases, holotransferrin causes a slight decrease in cell count and increase in apoptosis when compared with those of non-treated controls ($\chi^2=4.5, df=1, p<0.035$).

Sample images of apoptotic Molt-4 cells are shown in Figure 3. DHA induced mainly apoptosis in the cells. No necrosis was detected.

**Discussion**

Molt-4 cells have been shown to undergo apoptosis (17) and necrosis (18). Both types of cell death can be analyzed by DNA diffusion assay (13). To explore the mode of action by which artemisinin kills cancer cells, we used DHA, a more water soluble and more potent analog of artemisinin in cell
cultures. We observed a rapid drop in cell numbers and a rise in percentage of apoptotic cells in Molt-4 cell cultures treated with DHA. A further enhancement was observed in cells exposed to a combination of holotransferrin and DHA.

Rapid cell elimination has been reported in Molt-4 cultures (7) and in breast cancer cells (9) after treatment with holotransferrin and DHA. However, the mode of this cell elimination was not known. Using the DNA diffusion assay, we have shown that most cells undergo apoptosis and are rapidly eliminated. Since cell death and elimination of dead cells occurs rapidly, only a maximum of 27.5% apoptotic cells could be seen. We did not observe any necrosis. Our results are in accordance with the earlier findings of Sadava et al. (19) who showed, using the TUNEL assay, the induction of only apoptosis by artemisinin in a human small cell lung carcinoma cell line.

DHA and other analogs of artemisinin carry an endoperoxide group and generate free radicals in the presence of iron (3). These carbon-based radicals may damage cellular macromolecules including DNA, leading to apoptosis. The finding that pretreatment with holotransferrin significantly enhanced the apoptosis-inducing effect of artemisinin supports the notion that iron-induced free radical formation plays a major role in the cytotoxicity of artemisinin. Schmuck et al. (20), using DHA and artemisinin in primary neuronal cell cultures from fetal rat brain, have shown that the neurotoxicity of these compounds is mediated by production of reactive oxygen species, leading to damage to cellular macromolecules and inhibition of mitochondrial functions. Also, artemisinin alkylates several cellular proteins (21, 22), thus it is also possible that this may interfere with certain cellular metabolic pathways necessary for cell survival.

DHA causes rapid cell death and more than 95% cells were eliminated from cultures at the 8 h time point. As only 27.5% cells were going through apoptosis at 8 h, it may be concluded that dead cells are rapidly disintegrated in the cultures. This phenomenon may be advantageous in cancer therapy as it may lead to rapid reduction of tumor size.
One primary advantage of artemisinin therapy could be the compound's ability to induce apoptosis exclusively. Artemisinin was not observed to induce necrosis, which indicates that its action may not be associated with enhanced inflammatory reactions. Necrotic cells release cytokines after lysis and the resulting necrotic debris attracts inflammatory cells, leading to tissue destruction (23-24).

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References