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THE DUAL EFFECT OF CURCUMIN ON ETOPOSIDE ACTION IN LEUKEMIC AND HEALTHY BONE MARROW CELLS OF RATS WITH ACUTE MYELOID LEUKEMIA

Abstract: The effect of curcumin, a promising anticancer agent, on the action of certain cytostatic drugs, including etoposide, is not well understood. This paper examines the effect of curcumin on etoposide action in leukemic and normal bone marrow cells *in vivo* conditions. The experimental model used was Brown Norway rats with a transplantable acute promyelocytic leukemia. Leukemia was induced by intravenous injection of BNML (Brown Norway Myeloid Leukemia) cells. Curcumin was administered by oral gavage (200 mg/kg) for 23 consecutive days and etoposide was used intraperitoneally (50 mg/kg) for the last three days of the experiment. Control leukemic and healthy rats received the solvent for the tested compounds only. Curcumin significantly reduced the number of leukemic promyelocytes in the bone marrow of BNML rats in comparison to the leukemic control. Treatment with curcumin plus etoposide led to a decrease in the number of promyelocytes to the normal values occurring in healthy individuals. In contrast, the percentage of the normal precursors of granulocytes ($p < 0.001$) and erythrocytes ($p < 0.001$) increased significantly in comparison to the group treated with only etoposide. The results of the study indicate that curcumin may protect healthy myeloid cells against the cytotoxic effect of etoposide and potentiate the antileukemic action of this anticancer drug.

Key words: curcumin, etoposide, acute myeloid leukemia, bone marrow, rats.

INTRODUCTION

Curcumin, is the main polyphenol derived from rhizomes of the plant *Curcuma longa*. Curcumin exhibits a number of biological activities including anti-inflammatory, antioxidant, metabolism regulating, antifungal, antiparasitic, antibacterial and anticancer [1–4]. It has potent anticancerous activity in the cells of both solid tumors and leukemias [5]. The anticancer effect of curcumin depends on stimulation of apoptosis in malignant cells and inhibition of their proliferation [6, 7]. Curcumin may potentially inhibit the growth of leukemia cells by interfering in epigenetic phenomena. It unlocks hypermethylation which inactivates tumor suppressor genes in acute myeloid leukemia (AML) cells, leading to inhibition of leukemic cell growth *in vitro* and *in vivo* [8].

On the other hand, curcumin demonstrates low toxicity in normal cells [9]. It has also ability to protect normal cells from the toxic effects of chemotherapy [5]. It is believed that one of the reasons for such contrasting actions of curcumin are the differences in metabolism between cancer and normal cells [10]. For example, tumor cells are characterized by the preponderance of glycolysis over the aerobic metabolism and they also produce a lot of lactate, leading to acidosis in an extracellular environment acting as an immunosuppressive and promoting tumor growth [11]. Curcumin can reverse the altered metabolism of cancer cells by affecting the expression of glucose transporters and the production of lactate [12]. Cancer cells often have a high level of free radicals, in contrast to their low levels in normal cells [13]. Curcumin may exert increased prooxidant activity in the presence of a high level of free radicals, which are often present in tumor cells, and can stimulate these cells to apoptosis [14–16]. All the traits make a curcumin a promising anticancer compound that could be used in combination with conventional cytostatic drugs to protect healthy cells and/or enhance the therapeutic effects of anticancer drugs in cancer cells.

Other studies have shown that curcumin can exert a synergistic effect with cytostatics such as 5-fluorouracil, vincristine, paclitaxel, cisplatin or etoposide in cancer cells [5]. On the other hand, there are reports demonstrating an opposite antagonistic effect of this polyphenol with etoposide [17].

The effect of curcumin on the action of etoposide in cancer cells is, therefore, not well understood. Etoposide is used to treat certain solid tumors, as well as leukemias and lymphomas [18]. Previously, our own *in vitro* studies have demonstrated a synergistic effect of curcumin with etoposide in a rat myeloid leukemia LT-12 cell line [19]. These results require confirmation in an appropriate *in vivo* model.

The aim of this study was to determine the effect of curcumin on the action of etoposide in leukemic and normal bone marrow cells of Brown Norway rats with acute promyelocytic leukemia.

MATERIALS AND METHODS

Male Brown Norway (BN/CrlCmd) rats were purchased from the Animal Center, Polish Academy of Sciences Medical Research Center (Warsaw, Poland). The rats were kept under standard conditions of 22°C and 50–60% humidity, with a 12:12 h light-dark cycle. They were fed a synthetic pellet diet without plant polyphenols (Morawski Label Feed, Kcynia, Poland) and had free access to water.

BNML was induced in BN rats by intravenous inoculation of spleen-derived BNML cells (1×10^6 cells in 0.5 ml of sterile PBS injected into the tail vein). Cells were provided by Professor A.C.M. Martens, Utrecht University, the Netherlands [20]. Experiments were performed in accordance with animal testing regulations, under a license granted by the Jagiellonian University Ethical Committee.

BNML is a transplantable myeloid leukemia originally induced by several injections of dimethylbenzoanthracene [20]. This leukemia is characterized by a slow progression and constant rate of growth. Similarities between BNML and human AML include the sensitivity of rat leukemia cells to chemotherapy, inhibition of normal hematopoiesis and growth pattern [21]. This model was used to optimize drug dosage and to test the combinations of drugs used in AML. BNML rats were also used to test the methods of detection and treatment of minimal residual disease (MRD) [21].

The rats were divided into four groups of eight animals per group. Curcumin (Sigma-Aldrich Co. St. Louis, Missouri, USA) was administered by oral gavage at a dose of 200 mg/kg b.w. The dose of curcumin per rat was dissolved in 100 µl of DMSO Sigma-Aldrich Co. (St. Louis, Missouri, USA) and then diluted in 400 µl of corn oil Sigma-Aldrich Co. (St. Louis, Missouri, USA). Curcumin was administered once a day from the second day after the inoculation of BNML cells, until day 23 of the experiment. The doses of curcumin was chosen based on previous studies in which this polyphenol significantly increased apoptosis of cancer cells *in vivo* [22]. Etoposide (Bristol-Myers Squibb, Sermoneta, Italy) was administered intraperitoneally at a dose of 50 mg/kg b.w. once a day for the last three days of the experiment. The dose of etoposide was chosen based on the study by Turner *et al.* [23], in which this chemotherapeutic agent induced a significant cyto-genotoxic effect in the bone marrow of mice. Control BNML and healthy rats were treated with vehicles only.

The rats were killed by cervical dislocation under vetbutal anesthesia. The right femur was excised immediately after euthanasia and the bone was split longitudinally. Bone marrow was extracted from the femoral cavity using a small paintbrush moistened with FBS (Population Association of America, Pasching, Austria) and smears were prepared on glass slides. Smears were dried at room temperature, and then they were stained using May-Grünwald and Giemsa stain (Stamar, Gliwice, Poland). Stained smears were analyzed by counting approximately 500 cells/slides on three slides from each rat using an Olympus CX-40 microscope (Olympus, Tokyo, Japan).

Statistical significance between groups was determined using the one-way analysis of variance (ANOVA) and Tukey *post hoc* test. Data were expressed as mean ± standard deviation (SD).

RESULTS

The predominant cells in the bone marrow of BNML rats were leukemic promyelocytes, which prevailed over other granulocytic precursors and mature granulocytes and other bone marrow cells, such as erythroid cells, lymphocytes and monocytes (Table 1, Fig. 1). A significant decrease was observed in the percentage of

Table 1

Percentage of cells isolated from bone marrow of healthy control and leukaemic rats.

Group of rats	Promyelocytes (%)	Granulocytic precursors (myeloblasts, myelocytes, metamyelocytes, bands) (%)	Erythroid nucleated cells (%)	Mature granulocytes (%)	Monocytes (%)	Lymphocytes (%)
H	4,0 ± 1,6^{e}	15,6 ± 0,6^{a}	34,7 ± 1,6^{a}	33,0 ± 2,2^{a}	2,0 ± 0,8^{a,c}	18,2 ± 2,9^{a,c}
B	60,1 ± 4,1^{c}	6,7 ± 1,5^{b}	2,8 ± 0,8^{b}	8,6 ± 2,0^{b}	1,0 ± 0,1^{a,c}	12,2 ± 1,3^{b,c}
C	27,3 ± 4,0^{d}	10,9 ± 1,2^{c}	5,8 ± 0,9^{b}	14,0 ± 3,6^{b,c}	0,3 ± 0,5^{a}	6,6 ± 2,9^{b}
E	11,6 ± 1,7^{b}	3,5 ± 2,4^{d}	5,0 ± 2,8^{b}	20,0 ± 4,9^{c,d}	0,5 ± 0,7^{a}	24,5 ± 4,3^{a}
C+E	7,8 ± 2,6^{b,e}	11,9 ± 2,4^{a,c}	17,4 ± 3,5^{c}	27,0 ± 9,2^{a,d}	2,5 ± 1,7^{b,c}	22,8 ± 7,0^{a}

H, Healthy control rats; B, control leukaemic rats, C, leukemic rats treated with curcumin (200 mg/kg b.w.); E, leukaemic rats treated with etoposide (50 mg/kg b.w.); C+E, leukemic rats treated with curcumin (200 mg/kg b.w.) and etoposide (50 mg/kg b.w.). Data are presented as the mean percentage of each cell population ± standard deviation of the mean. Means having different signs (a, b, c, d) in the same column differ significantly, $p < 0.05$

normal granulocytic precursors ($p < 0.001$) and nucleated erythroid cells ($p < 0.001$) in comparison to healthy individuals (Table 1). The decrease in granulocytic precursors correlated with a decrease in the number of mature granulocytes ($p < 0.001$) in BNML rats compared to healthy individuals (Table 1). However, insignificant differences were observed in the ratio of lymphocytes and monocytes between the group of leukemic and healthy rats.

Etoposide treatment led to a significant decrease in the number of leukemic promyelocytes ($p < 0.001$) and normal granulocytic precursors (myeloblasts, myelocytes, metamyelocytes and bands) ($p < 0.05$) compared to the control leukemic rats (Table 1). In contrast, the percentage of mature granulocytes ($p < 0.01$) and lymphocytes ($p < 0.001$) significantly increased after etoposide treatment in comparison to control leukemic rats (Table 1). These mature cells dominated in the bone marrow of etoposide-treated rats (Fig. 1). However, there were insignificant differences in the amount of nucleated erythroid cells and monocytes between the group treated with etoposide and control leukemic rats.

The administration of curcumin also led to a significant decrease in the percentage of leukemic promyelocytes ($p < 0.001$), with a concomitant significant increase in the percentage of normal granulocytic precursors ($p < 0.05$) compared to control leukemic rats (Table 1). A clear, although insignificant, trend could be observed towards an increase in the percentage of nucleated erythroid cells and mature granulocytes in curcumin-treated rats compared to the control. There was also an insignificant change in the number of monocytes and lymphocytes after curcumin administration in comparison to control leukemic rats (Table 1).

It should be noted that a substantial percentage of death cells were observed in the bone marrow of curcumin or etoposide experimental groups. In comparison to other groups of rats, apoptotic bodies were most common in the group treated with etoposide (Fig. 1).

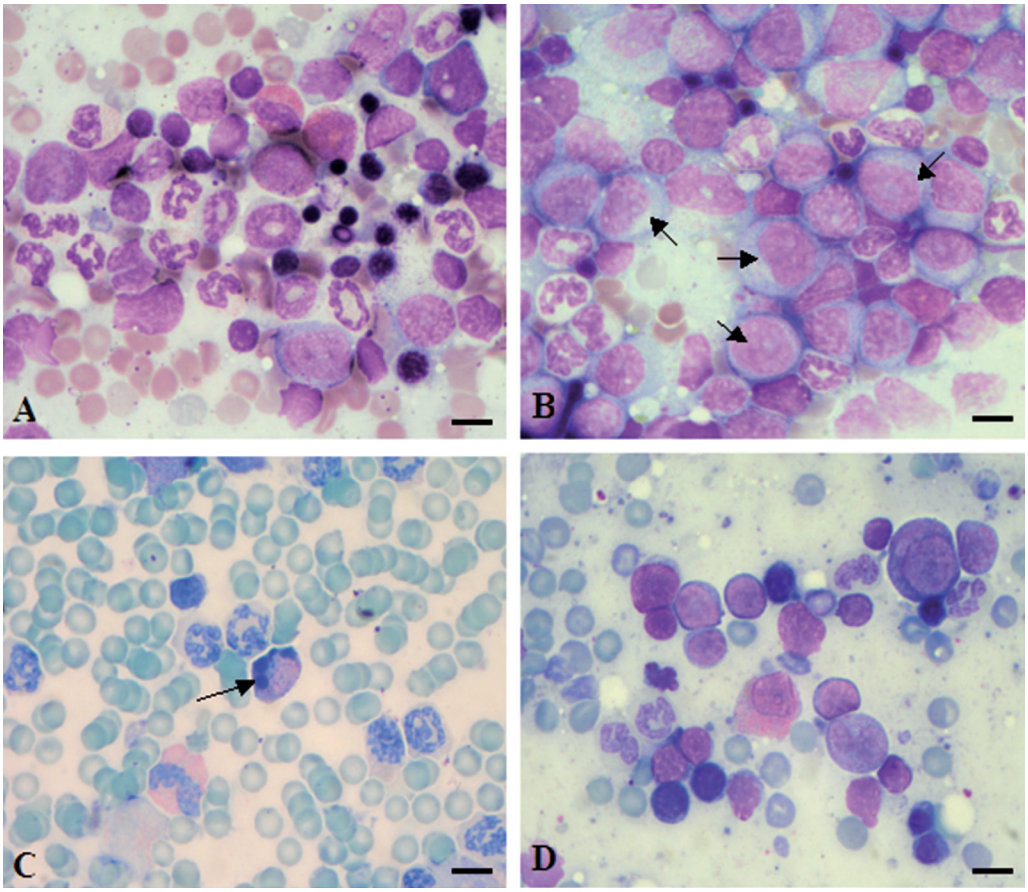

Fig. 1. Bone marrow smears of rats.

(A) Healthy control rat. (B) Control leukemic rat. Predominant cells are leukemic promyelocytes (arrows) (C) Leukemic rat treated with etoposide (50 mg/kg b.w.) for 3 days, from 20 day of leukemia development. Predominant cells are mature granulocytes. Arrow indicates apoptotic cell. (D) Leukemic rat pretreated with curcumin (200 mg/kg b.w.) for 22 days, followed by co-administration of etoposide (50 mg/kg b.w.) for the last 3 days of experiment. Bone marrow precursor cells are visible. May-Grünwald-Giemsa stain. ×1000. Barr = 10 µM.

Administration of curcumin both before and during etoposide treatment led to a further but insignificant decrease in the proportion of promyelocytes in comparison to the etoposide-only group. The number of promyelocytes seems to return to normal values in curcumin plus etoposide treated rats, because there is an insignificant difference between these group of rats and healthy subjects in

terms of the proportion of promyelocytes. In contrast, the number of granulocytic precursors ($p < 0.001$), nucleated erythroid cells ($p < 0.001$) and monocytes ($p < 0.05$) significantly increased in rats co-administered with curcumin and etoposide in comparison to the etoposide-only group (Table 1, Fig. 1). Importantly, the percentage of normal granulocytic precursors and mature granulocytes did not differ significantly between rats treated with curcumin plus etoposide and the healthy controls.

DISCUSSION

A major problem in the treatment of AML is still the low level of effectiveness of chemotherapy which is caused by the resistance of leukemia cells to conventional drugs [24, 25], and the side effects, such as myelotoxicity, which limit its use [26].

Neutropenia occurring in newly diagnosed patients with AML may worsen during the treatment and then there is an increased risk of bacterial infections, which in turn adversely affect the prognosis [26].

This paper presents for the first time that curcumin has a synergistic effect with etoposide in acute myeloid leukemia cells, while protecting normal hematopoietic cells from the cytotoxic effects of etoposide *in vivo*. Leukemic cells co-exist with normal cells of myeloid lineages in BNML rats. It is, therefore, a good model for the study of the effects of curcumin and etoposide on cancer cells and their normal counterparts in the organism.

Treatment with etoposide led to a significant decrease in the number of leukemic promyelocytes, and the bone marrow pattern indicated myelosuppression. The mature form of granulocytes and lymphocytes prevailed and dramatically decreased the number of progenitor cells of normal hematopoiesis (granulocytic and erythroid precursors), indicating a strong inhibition of cell cycle progression by etoposide. Probably, the reason for the strong myelotoxicity caused by etoposide in proliferating bone marrow cells is their high sensitivity to DNA damage in the presence of increased activity of topoisomerase II [27]. Etoposide inhibits topoisomerase II DNA-cleavable complexes, leading to the formation of DNA strand breaks, including the most lethal DNA double-strand breaks (DSBs) [28], resulting in apoptosis or necrosis, and consequently myelosuppression. It is believed that the myelotoxicity of etoposide corresponds, to a large extent, with the etoposide phenoxyl radicals. These radicals can cause a decrease in glutathione levels and exacerbate the oxidative damage to DNA and DSBs [28, 29]. On the other hand, oxidative damage to mitochondrial membrane phospholipids can result in a loss of their integrity, which also leads to cell apoptosis.

It is known that the myelosuppressive effect of etoposide limits the use of this chemotherapeutic agent in humans [30]. The limitation of this serious complication associated with etoposide therapy may enable an increase in etoposide dosages and thereby improve the effectiveness of the treatment.

Slightly different changes occurred in the bone marrow of BNML rats treated with curcumin throughout the period of leukemia development. Treatment with curcumin from the initial stage of leukemia resembles the situation when the disease relapses after remission.

The percentage of promyelocytes decreased significantly in the curcumin-treated group, although to a lesser extent than after treatment with etoposide. In contrast, the percentage of granulocytic precursors increased significantly compared to control rats with leukemia. Therefore, the results indicate the cytotoxic action of curcumin in promyelocytic leukemia cells and its protective activity in healthy myeloid cells. Other studies have shown that polyphenols may increase oxidative stress in cancer cells with elevated levels of free radicals [14, 31]. The results of previous studies have also demonstrated increased oxidative stress and high levels of DSB and oxidative damage to DNA in tissues of BNML rats that correlated with the high levels of leukemic cells [16]. Curcumin may be oxidized by a myeloperoxidase into phenoxyl radicals in myeloid leukemic cells [32], leading to increased oxidative stress and, consequently, to apoptosis.

After co-administration with curcumin and etoposide, the percentage of bone marrow cells is normalized, and this group is most similar to healthy animals. The percentage of granulocytic precursors, including promyelocytes, mature granulocytes and monocytes, is insignificant in this group compared to healthy controls. This observed phenomenon may result from the fact that the rats treated with curcumin before the administration of etoposide had more normal hematopoietic precursors than animals only treated with etoposide. Other similar results have shown that curcumin at a dose of 100 mg/kg b.w. provided protection against mitomycin C-induced myelosuppression in mice with a xenogenic transplantable model of breast cancer, while causing cytotoxic effects in cancer cells [33].

The results of the present study indicate that curcumin alone does not completely eliminate cancer cells in BNML rats, but it can protect against myelosuppression during the use of etoposide. Study of the mechanisms of etoposide interaction with the examined polyphenol may be useful in the prevention of side effects caused by this cytostatic drug in normal proliferating cells.

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CONFLICT OF INTEREST STATEMENT

None declared.

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