Abstract. Background: (−)-Epicatechin (EC) induces oxidative DNA damage in HL-60 cells. The association between genotoxic and apoptotic/necrotic effects of EC was studied in rats with acute myeloid leukaemia. Materials and Methods: Healthy and leukaemic rats were given EC by oral gavage at a dose of 40 mg/kg body weight for 22 consecutive days. Bone marrow cells were subjected to analysis of DNA damage by the comet assay and apoptosis by flow cytometry. Results: EC significantly increased DNA strand breaks in bone marrow cells of leukaemic animals but it did not exert such an effect on healthy rats. EC action led to necrosis of leukaemia cells but it did not induce apoptosis of these cells in comparison to the controls. Conclusion: EC has genotoxic and necrotic effects which may have utility in anticancer therapy against acute myeloid leukaemia.

Studies carried out on animal models and population-based case–control studies indicate the chemopreventive effect of green tea extracts in relation to various types of neoplasms (1-3). Green tea administered to mice in a non-Hodgkin’s lymphoma model prevented the development of tumours by 50% and significantly inhibited cancer cell proliferation (4). The most important active ingredient of green tea is epigallocatechin-3-gallate (EGCG), whose chemopreventive properties have been proven in vitro and in vivo (5, 6).

Although the effect of epicatechin on cancer cells is not widely known yet, it is believed that the main mechanism of the anticancerous and proapoptotic action of some plant polyphenols is via pro-oxidative activity which depends on the redox state of a cell (7). These polyphenols are characterized by having a selective, pro-oxidative action in neoplastic cells producing large amounts of free radicals and do not exert such activity in healthy cells without any disturbances in antioxidative defence (8, 9-11). EGCG, the most abundant polyphenol among the green tea catechins, exerts a pro-oxidative effect and induces apoptosis in the cells of HL-60 line, derived from human promyelocytic leukaemia with high myeloperoxidase (MPO) activity (12). It has been proven that MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) suppresses the apoptotic effect of EGCG on HL-60 cells. However, overexpression of MPO activity led to an increase in reactive oxygen species (ROS) production and sensitisation of EGCG-resistant K562 cells to apoptosis induced by catechin (10). Moreover, EGCG induces the generation of H2O2, and subsequently the hydroxyl radical, which contributes to apoptosis of leukaemia cells with high MPO activity.

Several studies have indicated that EC protects some neoplastic cells from oxidative stress and oxidative DNA damage (13, 14). It exerts the opposite effect on cells of the HL-60 line, which show high MPO activity. Being a very effective substrate for MPO, EC may be converted to phenoxy radicals, which induce oxidative modification of DNA bases (15, 16). Therefore, it can be supposed that EC may eliminate the myeloid leukaemia cells that show high MPO activity, similarly to the case of EGCG.

The aim of the present study was to determine whether EC influences in vivo DNA damage of bone marrow cells from leukaemic rats. The study also investigated whether the DNA-damaging effect of EC on these rats results in apoptosis or necrosis of the leukaemia cells.

Materials and Methods

Animals. Brown Norway rat myeloid leukaemia (BNML) rats were used. BNML rats are an appropriate model for the study of acute myeloid leukaemia (17). There are many similarities of BNML to...
human acute myeloid leukaemia (AML); for example, BNML cells react to chemotherapeutic agents similarly to AML cells, and normal haematopoesis is strongly suppressed during development of rat leukaemia (17-19).

Four-month-old male Brown Norway (BN/CrlCmd) rats (310.3±4.2 g) were obtained from the Animal Center, Polish Academy of Sciences Medical Research Center (Warsaw, Poland).

BN rat myeloid leukaemia was developed after intravenous inoculation with 10⁶ splenic-derived leukaemia cells (kindly provided by Professor A.C.M. Martens, Utrecht University, The Netherlands) in phosphate buffered saline (PBS) (PAA, Pasching, Austria). The experiments were performed in accordance with legal requirements, under a licence granted by the Jagiellonian University Commission of Ethics.

Treatment with EC. Initial studies were performed in order to determine the dose of EC (Sigma–Aldrich) to be used for the main study investigations. Groups of four BNML rats were each given EC by gavage at a dose of 20 or 40 mg/kg b.w., dissolved in 0.5 ml of water for 22 consecutive days. Control animals were given plain water only. Behaviour, weight and general condition were monitored. A dose of 40 mg/kg b.w. was used in the remaining investigations, based on the study of DNA damage in bone marrow cells.

The main experiment was performed on healthy and leukaemic rats; which were divided into two groups, control and experimental. The rats in the experimental group were given EC by oral gavage (40 mg/kg b.w.) once a day for 22 consecutive days (for the leukaemic rats, from the second day after inoculation), while those in the control group were given water only during the same period. Each of the control and experimental groups had six healthy and eight leukaemic animals.

Bone marrow samples. The rats were killed two hours after the final EC administration. The femur was removed and bone marrow cells were flushed with 1 ml fetal bovine serum (FBS) (PAA, Pasching, Austria) and then washed once in PBS. The cell suspension was used directly in the comet assay. In order to analyse cells by flow cytometry, erythrocytes were lysed with red blood cell lysing buffer (Sigma–Aldrich).

Comet assay. The extent of DNA damage in the bone marrow cells was determined by alkaline single-cell gel electrophoresis (pH=13) as previously described (20). Formamidopyrimidine [fapy]-DNA glycosylase (Fpg, 1 unit/slide) (Sigma–Aldrich) was used to disclose the oxidised purine bases (Fpg-labile sites) The electrophoresis was carried out for 30 min at 0.74 V/cm and 300 mA. The slides were then stained with propidium iodide (2.5 μg/ml; Sigma–Aldrich) and analysed using fluorescence microscopy (Olympus CX-40; Olympus, Tokyo, Japan). The Comet Score™ (TriTek Corporation, Sumeduck, VA, USA) image analysis system was used for the quantification of DNA damage. The percentage of DNA in the tail was measured. At least two slides per rat, with 100 randomly selected comets per slide, were analysed.

Detection of BNML cells and analysis of apoptotic and necrotic cell death. Samples of 1×10⁶ bone marrow cells were incubated for 45 min on ice in 100-μl PBS with 5% FBS, containing 10% mouse ascites primary anti-RM124 antibody (kindly provided by Professor A.C.M. Martens), which recognises the specific epitope of the leukaemia cells (21). The secondary antibody used was goat anti-mouse monoclonal antibody conjugated to R-phoerythrin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). To evaluate the nonspecific binding of a secondary antibody to B-lymphocytes, the cells were also incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45R monoclonal antibody (BD Biosciences Pharmingen, San Diego, CA, USA). Subsequently, the cells were stained with 2 μl Annexin V-APC (An) (BD Biosciences) and 2 μl 7-aminoactinomycin D (7AAD), both from BD Biosciences), in the dark for 15 min. The cells were analysed by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (both from BD Biosciences). Apoptotic cells were defined as An-positive and 7AAD-negative, while necrotic and/or late apoptotic cells were An- and 7AAD-positive, necrotic cells were 7AAD-positive and An-negative. Results were expressed as a percentage of apoptotic or necrotic cells.

Results

**DNA strand breaks and oxidative modification of purine bases in healthy and leukaemic rats.** To determine whether the oxidative stress present in the tissues of BNML rats (22) is accompanied by oxidative DNA damage, the extent of oxidization of purine bases in the bone marrow cells of healthy and leukaemic rats was measured using the comet assay. The analysis revealed that BNML rats showed significantly (p<0.01) higher extent of oxidization of purine bases (Fpg-labile sites) in comparison to the group of healthy animals (Figure 1). The study of DNA strand breaks revealed a lack of significant differences in the extent of this damage in bone marrow cells between healthy and leukaemic rats (Figure 2).

The influence of EC on DNA damage. The initial study showed that EC at a dose of 20 mg/kg b.w. did not significantly influence DNA strand breaks in bone marrow cells of BNML rats (12.4±2.6% of tail DNA) in comparison to the control group (11.6±1.4% of tail DNA). However, a dose of 40 mg/kg b.w. of EC significantly increased the proportion of DNA strand breaks in these cells (19.8±1.1% of tail DNA). Thus, the dose of 40 mg/kg b.w. of EC was used in the rest of the study. To take into account that leukaemic rats are exposed to more oxidative DNA damage in bone marrow cells than healthy animals, the study determined whether EC exerts a different influence on the proportion of DNA damage (oxidised purine bases and strand breaks) on these cells of both rat groups. EC (40 mg/kg b.w.)
did not exert a genotoxic effect on the bone marrow cells of healthy rats because the extent of DNA strand breaks and Fpg-labile sites did not change significantly in this experimental group (Figures 1 and 2). A significant increase in DNA strand breaks was observed in cells isolated from the bone marrow of BNML EC-treated rats (Figure 2). However, EC did not significantly modify purine bases in the cells of leukaemic rats in comparison to the respective control group (Figure 1).

Influence of EC on the number of leukaemic cells, their apoptosis and necrosis. To determine whether the DNA damage induced by EC in the bone marrow influences the survival of BNML cells, the percentage of apoptotic and necrotic cells was measured. The results indicate that EC (40 mg/kg b.w.) induced selective necrotic cell death in BNML cells. In the EC-treated rats, there was a significant increase in the percentage of 7AAD^+An^− BNML cells in comparison to the control group (p<0.001) (Figure 3). EC did not exert a significant influence on the percentage of apoptotic cells in the examined leukaemic animals (Table I).

To study whether necrotic cell death induced by EC reduces the proportion of BNML cells in the bone marrow, the percentage of these cells was evaluated using flow cytometry. The analysis revealed that the percentage of BNML cells was unchanged in the group of EC-treated rats in comparison to untreated leukaemic ones (Table I).

A significant negative correlation existed between the percentage of BNML cells and necrosis of these cells in the EC-treated leukaemic rats (Figure 4).

Table 1. The percentage of leukaemia cells (BNML) and apoptotic cells in the bone marrow of Brown Norway rats treated with (−)-epicatechin for 22 days. Data are represented as mean±standard error of the mean. Statistical significance was set at p<0.05.

<table>
<thead>
<tr>
<th>Group of rats</th>
<th>L</th>
<th>L+EC</th>
</tr>
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<tbody>
<tr>
<td>Dose of EC (mg/kg b.w.)</td>
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<td>40</td>
</tr>
<tr>
<td>An^+7AAD^− cells, %</td>
<td>9.2±1.4</td>
<td>19.2±6.9^*</td>
</tr>
<tr>
<td>An^+7AAD^+ cells, %</td>
<td>12.9±3.2</td>
<td>8.2±1.7^*</td>
</tr>
<tr>
<td>BNML cells, %</td>
<td>35.6±5.8</td>
<td>19.7±6.3^*</td>
</tr>
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L, Leukaemia cells of control rats; EC, leukaemia cells of epicatechin-treated rats; ^*statistically non-significant difference vs. L.
Discussion

Cancer cells often produce more ROS than their healthy counterparts (23, 24). A previous study demonstrated the existence of oxidative stress in the spleen and brain of BNML rats (22). The present study showed that the bone marrow cells of leukaemic rats suffer more oxidative DNA damage in purine bases in comparison to the healthy animals.

In vitro research has indicated that EC action in leukaemia cells may depend on the redox state. This polyphenol induces the formation of 8-oxoguanine in HL-60 cells (16) with constitutively high MPO activity. The genotoxic action of this compound in HL-60 cells may involve the induction of DNA damage through its fenoxyl radicals and/or generation of H2O2 in the redox cycle, which leads to oxidative DNA damage (16). In contrast, in human hepatoma cell line (HepG2) and healthy fibroblasts, EC does not cause such DNA damage but protects those cells against different pro-oxidative agents (25, 26). EC, as a substrate of MPO, may be able to kill the myeloid leukaemia cells. It has been proven that the cytotoxicity induced by conventional anticancer drugs positively correlates with the expression of MPO in the HL-60 cell line (27). Moreover, the percentage of MPO-positive blast cells is a prognostic factor in AML patients (27). In the present study, EC administration in rats did not induce oxidative damage of purine bases in DNA. However, the examined polyphenol induced DNA strand breaks in the bone marrow cells of leukaemic rats without any adverse effect on healthy rats. The lack of any EC effect on oxidative DNA damage in bone marrow cells of leukaemic rats indicated that the mechanism of its action differs from that in HL-60 cells.

Leukaemia cells may be more susceptible to DNA strand breaks induced by EC than healthy cells because of their genetic instability. It is believed that the number of mutations in oncogenes increases in myeloid leukaemia cells (28-30). Activated oncogenes generate ROS leading to an increase in genomic instability of these cells (31-33). Moreover, cancer cells often possess dysregulated mechanisms of DNA repair, resulting in increased susceptibility to DNA damage under the influence of genotoxic agents (34). The mechanism of genotoxic action of EC may also depend on the inhibition of topoisomerase I expression, which was observed both at mRNA and protein levels (14).

The present study demonstrated that EC induces necrosis of BNML cells and this negatively correlates with the percentage of these cells. Although the examined polyphenol did not induce apoptosis of leukaemic cells, there is a tendency for an increase of early apoptotic cells. These results agree with the study of Iwasaki et al. (35), which revealed that EGCG induces necrosis-like cell death in chronic myelogenous leukaemia cell line K562. That study also suggested that EGCG does not modulate the apoptotic pathway in these cells by caspase-independent induction of cell death.

In the present study, EC induced neither necrosis nor apoptosis of non-leukaemia bone marrow cells. Similar results were obtained by Iwasaki et al. (35), who demonstrated that EGCG exerts minimum toxicity on healthy human peripheral blood mononuclear cells.

Necrosis is an immunogenic process; therefore antigens of necrotic neoplastic cells intensify the immune response against tumour (36, 37). Thus, it is to be supposed that EC will be helpful in killing cancerous cells which are resistant to apoptosis, while inducing no toxicity in healthy cells.
Despite the genotoxic effect and the induction of necrosis under the influence of EC, no significant decrease of the percentage of BNML cells was observed in the present study. The reason for such a low efficiency of EC may be its relatively low bioavailability. Unpublished observations have indicated that the concentration of EC in plasma of rats is as low as 7 μM. It is, therefore, suggested that an increase of EC bioavailability by modification of its structure or intravenous administration may contribute to increasing its effect.

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References
