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Baicalin from the extract of *Scutellaria baicalensis* affects the innate immunity and apoptosis in leukocytes of children with acute lymphocytic leukemia

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ABSTRACT

Scutellariae Radix (root of *Scutellaria baicalensis*) has a long history of application in traditional and in modern herbal medications. The major components of Scutellariae Radix are baicalin, baicalein, wogonoside and wogonin. Accumulating evidence demonstrates that *Scutellaria* has immunomodulatory effects and possesses compelling anticancer potential.

Treatment of peripheral blood leukocytes (PBLs) with *Scutellaria* extract (SBE) enriched in baicalin, reduced viability of PBLs obtained from patients with acute lymphoblastic leukemia (ALL). SBE had no impact on the survival of healthy, control leukocytes. The immune system modulation by SBE resulted in increased production of IFN-γ in PBLs, and reduced TNFα and IL-10 production in bone marrow cells (BMC), in ALL patients. SBE stimulated the nonspecific antiviral immunity, assessed by resistance of PBLs and BMC to vesicular stomatitis virus (VSV) infection. SBE showed pro-apoptotic activity in NALM-6 cell line (B-type human leukemia). The number of cells expressing annexin V increased from 6% in control cultures to 29% and 52% after treatment with 100 μg/ml and 200 μg/ml respectively. Increased percentage of apoptotic cells was observed when cells were treated with corresponding concentration of baicalin. SBE enhanced apoptosis of PBLs in BMC of leukemic children. The percentage of PBLs that underwent apoptosis and mean annexin V expression increased from 11% in the control to 17% and 24% for the doses of 100 μg/ml and 200 μg/ml respectively. Importantly, SBE did not induce apoptosis of PBLs in the healthy, control group.

1. Introduction

Cancer is now one of the most important medical problems in the world. Even with the discovery of many synthetic compounds with proven anticancer activity such therapies are usually expensive and not well tolerated by patients. Radiotherapy and chemotherapy, despite their beneficial proapoptic effect on neoplastic cells, exhibit significant toxicity for normal, healthy cells. Therefore nontoxic therapeutics of natural origins are an appealing alternative for treatment of cancer. An example of such therapeutics is flavonoids isolated from Scutellariae Radix (root of *Scutellaria baicalensis* Georgi) with their antioxidative, proapoptic activity, and anticancer potential [1–3]. According to the "cancer immunoediting" hypothesis the tumor immune response includes three phases [4]. In the first one, described as the elimination phase, the immune system can detect and eradicate tumor cells. Both the innate and the adaptive immunity are engaged in unspecific activity against pathogens as well as in anticancer activity [5]. Protective immune response observed in the "elimination phase" corresponds to the original concept of cancer immunosurveillance. According to this theory cancer cells are proficiently recognized and destroyed by the body's immune system. However, if the tumor cells are not eliminated, the equilibrium or escape phase proceeds and the tumor becomes well established. In the "equilibrium phase" tumor growth is controlled by the immune system but tumor cells are not completely eradicated. In the "escape" phase the tumor progresses and metastasizes [4,6]. The delicate balance between tumor cell growth and elimination may be disturbed by immune manipulations enhancing naturally occurring immunosurveillance. Flavonoids isolated from *S. baicalensis* Georgi have been widely investigated for their anticancer activities, general low toxicity and availability [1]. Scutellariae Radix contains four major flavones: baicalin, baicalein, wogonin and wogonoside. The extract

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used in this study was enriched in baicalin which is the major anticancer component of *Scutellaria*. Baicalin has been shown to have apoptotic activity against acute lymphocytic leukemia, lymphoma and myeloma cell lines [7].

Our current study focuses mainly on modulation of innate immune responses by *S. baicalensis* extract (SBE) enriched in baicalin. We also investigated the direct anticancer properties of SBE against not only the B cell leukemia cell line but also the leukocytes (PBLs) obtained from acute leukemia (ALL) patients. In our earlier studies we described a reaction of innate immunity expressed as the unspecific resistance of murine and human cells ex vivo to viral infection [8–12]. The resistance to viral infection was assessed with three different viruses: herpes simplex type 1 (HSV-1), encephalomyocarditis (EMCV), and vesicular stomatitis (VSV). These have a different structure, different mode of replication and different classification affiliation. The unspecific resistance of cells to virus infection depends upon, inter alia, the presence of endogenous cytokines (IFNs and TNFs). It has been shown that such resistance is individually differentiated and is influenced by age, and health status of blood donors. This indicates that resistance to viral infection represents one of the innate immunity reactions [13–16]. To characterize the degree of resistance, a test based on virus replication in freshly isolated peripheral blood leukocytes (PBLs) was designed [13]. The test utilizes bovine vesicular stomatitis virus (VSV) as an indicator virus to study PBL resistance to infection. Because VSV does not cause natural infection among the European human population there are no specific anti-VSV antibodies present in human sera. This excludes their participation in the maintenance of leukocytes' resistance [9,13]. Zaczyńska et al. showed that the whole population of PBLs expressed stronger resistance than fractions (adherent cells, leukocytes enriched in T or B lymphocytes, NK+ and NK− cells) and the level of resistance of the whole population of PBLs to VSV infection is the most informative and repeatable [17]. Recently VSV has drawn more interest due to construction of antiviral and anticancer vaccines [18,19]. As we stated above, the antiviral resistance of human leukocytes is important not only during viral infection, but also for the development of cancer. We have found that deficiency in antiviral resistance of PBLs is associated with failure of the induction of remission of bone marrow cells (BMC) after chemotherapy and survival time in adult patients with acute leukemia. Complete remission of BMC after chemotherapy was obtained in patients with PBLs resistant to VSV infection. In contrast, in patients with PBLs sensitive to infection, no remission of bone marrow cells was observed [20].

The effect of SBE in acute lymphoblastic leukemia (ALL) is still poorly understood; a limited anti-proliferative effect of SBE on primary human cells has been reported. The present study will not only investigate the direct anticancer properties of *S. baicalensis* extract (SBE), enriched in baicalin, in acute lymphoblastic leukemia (ALL) cell lines and primary patient-derived ALL leukocytes, but also provide a novel insight into the influence of baicalin on innate antiviral resistance of leukocytes isolated from children with acute lymphoblastic leukemia (ALL).

2. Materials and methods

2.1. Isolation of flavonoids from S. baicalensis

The official name of the dry extract of *S. baicalensis* (*S. baicalensis* radix) is "a complex of flavonoids isolated from the root of *S. baicalensis* (baicalin content not less than 65%)". It is used as an active substance in the medicinal product Baikadent (Marketing Authorisation Number 8865 issued by the Minister of Health in Poland). Baikadent and complex of flavonoids isolated from the root of *S. baicalensis* are produced by W.Z.Z. Herbapol SA (Poland) under Good Manufacture Practice requirements. The roots of *S. baicalensis* have to meet specified requirements. Preparation that was enriched in baicalin was obtained from fresh roots of *S. baicalensis* by extraction with water acidified with citric acid to pH 5. The final product was obtained by precipitation, filtration and drying. To prepare a stock solution, a sample of 10 mg of powder was suspended in 1 ml of RPMI 1640 medium. The complex of flavonoids was standardized with respect to baicalin content by analytical HPLC separation (LCMI system; Waters Chromatography Canada Inc.). The content of baicalin in the SBE was determined as 5.52 mg/ml (Fig. 1A and B).

2.2. Preparation of flavonoid suspensions for in vitro experiments

The stock solutions were made up from dry extract from roots of *S. baicalensis* which is described above. Dry powder was weighed using a sensitive scale (Radwag XA/2X). The powder was then brought to a concentration equal to 10 mg/ml using cell WFI grade H_2O (WFI Quality Water from EMD Millipore Chemicals, MA, USA) and stored in aliquots at −80 °C. To prepare the different concentrations of the extract for cell culture experiments, thawed stock was vortexed immediately before diluting into RMPI 1640 medium (IIET, Wroclaw, Poland) that contains 5% fetal bovine serum (FBS, Sigma-Aldrich, USA). After performing a cytotoxicity study on the PBLs of healthy volunteers and ALL patients, a concentration of 50 μg/ml was chosen for the study of innate immunity, and 100 and 200 μg/ml in apoptosis experiments. Control baicalin was obtained from ChromaDex, CA. Baicalin was used at concentrations of 8, 16, 32, and 64 μg/ml which correspond to the content of baicalin in SBE (25, 50, 100, and 200 μg/ml aliquots of SBE respectively). The endotoxin level in SBE was determined by The Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) QCL-1000TM assay (Lonza). The presence of endotoxin in samples was 0.6 U/ml.

2.3. The characteristics of groups enrolled to the study

Peripheral blood leukocytes (PBLs) were isolated from blood and the bone marrow of white Caucasian children (1–17 years old, median = 6) with acute lymphoblastic leukemia (ALL). Patients (n = 26) were treated at the Department of Bone Marrow Transplantation, Oncology and Hematology of the Wroclaw Medical University in 2008–2012. In all patients, the diagnosis and classification of risk groups for therapeutic and treatment were established according to up-to-date standard protocols adopted by the Polish Pediatrics Leukemia/Lymphoma Study Group (ALL BFM-90, ALL IC 2002). The percentage of undifferentiated cells in the bone marrow of the patients ranged from 49.5% to 99.50% with an average of 88.98%, and a median of 95%. The diagnosis was based on cytochemical examination and immunophenotyping of leukemic blasts. The control group included 16 patients ranging from age 1 to 17 years (median = 12) whose peripheral blood was collected while performing other tests. Based on medical interviews and physical examination acute infection and cancer were ruled out. This study has been reviewed, approved, and conducted in accordance with the guidelines of the Ethics Committee (KB-52/2009). A signed consent was taken from all participants of the study.

2.4. Cells

The *NALM-6* cell line (human, peripheral blood, leukemia, pre-B cell) was obtained from DSMZ (Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures). Cells were cultured in complete RPMI 1640 culture medium (IIET, Wroclaw, Poland) supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM L-glutamine (all from Sigma-Aldrich, USA). Cells were maintained at 37 °C and 5% CO_2 atmosphere. The *L929* cell line (ATCC CCL 1) — the murine fibroblast-like cell line and the *A549* cell line (ATCC CCL 185) — human lung carcinoma cell line were maintained in RPMI 1640 culture medium and Dulbecco medium (IIET, Wroclaw, Poland) respectively, supplemented with 10% fetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM L-glutamine. Human peripheral blood leukocytes (PBL) and bone marrow cells (BMC) were isolated according to the standard protocol from heparinized peripheral blood (10 U/ml) by