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Report: laboratory investigations

Cimicifuga racemosa extract inhibits proliferation of estrogen receptor-positive and negative human breast carcinoma cell lines by induction of apoptosis

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Summary

Hormone replacement therapy is contraindicated in women with breast cancer. Extracts from the rhizomes of *Cimicifuga racemosa*, have gained acceptance as a natural alternative for the treatment of menopausal symptoms. In the present study we investigated the antiproliferative activity of *C. racemosa* extracts (isopropanolic and ethanolic) on the estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB231 breast cancer cells by WST-1 assay. Down regulation of the proliferative activity and cell killing by isopropanolic and ethanolic extracts occurred in a clear dose-dependent response with a 50% growth inhibitory concentration of 54.1 ± 11.4 and $80.6 \pm 17.7\,\mu$g/ml in MCF-7 cells and of 29.5 ± 3.0 and $58.6 \pm 12.6\,\mu$g/ml in MDA-MB231 cells, respectively. Further, the mode of cell death was identified as apoptosis by microscopic inspection and confirmed by light scatter characteristics and by detection of Annexin V adherence to phosphatidylserine by flow cytometry. In addition, the involvement of activated caspases was supported by the cleavage of cytokeratin 18 detected with M30 antibody. Increases in the level of M30-antigen of about 4-fold and 2-fold over untreated controls were observed in *C. racemosa*-treated MCF-7 and MDA-MB231 cells. These results indicate that *C. racemosa* extract exerts no proliferative activity, but kills the estrogen receptor positive MCF-7 as well as estrogen receptor negative MDA-MB231 cells by activation of caspases and induction of apoptosis.

Introduction

Hormone replacement therapy (HRT) is commonly prescribed in menopausal treatment. Evidence of a link between breast cancer and postmenopausal HRT has been steadily accruing over the past few years. The recognition that naturally occurring 17β-estradiol is a weak genotoxic and mutagenic carcinogen and that prolonged exposure to endogenous estrogen is an adverse risk factor provided a plausible background for the association of breast cancer with HRT [1]. HRT is therefore contraindicated in estrogen-sensitive cancer patients. In recent years, the extract of the rhizomes of black cohosh *Cimicifuga racemosa* (CR) has been recognized as a rational choice for treatment and prevention of menopausal disorders [2, 3]. Herbal extracts typically contain a variety of constituents and therefore the precise mode of action of CR is not yet known. The binding of the constituents of CR to estrogen receptors [4, 5] could be involved in its pharmacological effectiveness. Other plant extracts used in the treatment of menopausal symptoms namely soy and red clover extracts as well as their abundant flavonoid genistein showed significant competitive binding to ER-α and β, but exerted estrogenic activity as opposed to the weak binding activity of CR extract to ER [6]. In addition, CR showed no estrogenic activity in the proliferation assay on MCF-7 cells and on gene expression using estrogen inducible MVLN cells, but antagonized these activities [7]. In a

randomized, double-blinded study of perimenopausal and postmenopausal women treated with isopropanolic aqueous CR no estrogenic effect could be found over a 24-week period [8]. Recently it was reported that CR-extract significantly inhibited the growth of breast MCF-7 estrogen receptor (ER)-positive cells, enhanced the inhibitory effect of tamoxifen and inhibited the estrogen-induced proliferation of MCF-7 cells adapted to estrogen-free medium using charcoal-stripped fetal calf serum [9]. Natural plant products have recently attracted attention because of their intriguing biological activities, especially for women seeking therapeutic alternatives. Such data suggest that CR could be a safe natural alternative for the treatment of menopausal symptoms in patients with ER-positive mammary cancer in contrast to soy or red clover products which seem to induce cancer cell proliferation under certain conditions [10–12]. Apoptosis is an essential regulatory mechanism and an imbalance between mitosis and apoptosis has broad ranging pathologic implications and has been associated with many autoimmune disorders, malignant tumors and viral infections [13]. Therefore natural compounds which can influence this balance between cell growth and programmed cell death are of growing interest. Apoptotic cell death involves a series of morphological and biochemical changes orchestrated by activated proteases belonging to the caspase family. Caspases are the central executioners of apoptosis and comprise a family of at least 14 members in mammalian cells [14]. One of their actions is the cleavage of the intermediate filament cytokeratin (CK) 18 in epithelial cells which can be recognized by the monoclonal antibody M30 [15]. The increase in the M30 activity correlates with increasing levels of caspases during apoptotic cell death.

The aim of our study was to evaluate the potential activity of an isopropanolic extract from black cohosh (iCR). We examined its cytotoxic effects and the mode of cell death (apoptosis) after treatment of estrogen receptor positive (ER^+) MCF-7 and estrogen receptor negative (ER^-) MDA-MB231 breast cancer cells. The effect of estrogens (17-β estradiol and genistein) as well as that of the non-steroidal partial antiestrogen tamoxifen were investigated. In the present study we show that the cell growth inhibitory effect of CR in both ER^+ and ER^- breast cancer cells could be related to its apoptosis-inducing activity as detected by the adhesion of Annexin V to phosphatidylserine on the outer leaflet of the cell membrane and by cell size scatter characteristics using flow cytometry, and that caspases are involved.

Materials and methods

Reagents

Cimicifuga racemosa extracts (CR) were provided by Schaper & Brümmer GmbH & Co.KG. Salzgitter, Germany. Two CR preparations were tested, an isopropanolic (B.Nr. 010720, iCR) and an ethanolic (B.Nr. 809220, eCR) extract. The concentrations of the extracts were 100 mg/ml (iCR) and 32.5 mg/ml (eCR) in relation to the dry residue. Concentrations of the total triterpene glycosides calculated as standard triterpene glycosides (27-deoxyactein) were 26 mg/ml (010720) and 9.46 mg/ml (809220), respectively. Alcohol concentration was between 50 and 60% (v/v). Tamoxifen citrate (TAM), 17 beta-estradiol (E2) from (Sigma, Buchs, Switzerland), genistein (4′, 5, 7-Trihydroxyisoflavone; GEN) and staurosporine (STP) from Alexis Corporation (Lausen, Switzerland), were used. The final concentration of ethanol and isopropanol in culture medium during CR treatment did not exceed 0.5% (v/v); therefore, the same concentration of both substances was present in control samples. This concentration did not affect the apoptosis or proliferation of the investigated cells. Cell proliferation reagent WST-1 was purchased from Roche Diagnostica (Rotkreuz, Switzerland).

Cell lines and culture conditions

Breast-adenocarcinoma cell lines MCF-7 (ER^+) and MDA-MB 231 (ER^-) from ATCC (Rockville, MD) were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/streptomycin (PenStrep) under a fully humidified atmosphere, 5% CO_2 at 37°C. The cytotoxicity and apoptosis assays were performed in DMEM medium without phenol red, containing, 5% charcoal stripped FCS (CCS, Sigma) and 0.2% PenStrep (test medium). In all experiments untreated and vehicle treated cells were included as controls. For experiments, cell were collected from subconfluent monolayers with trypsin/EDTA. The studies were carried out using cells from passages 3–7, and preincubated 24 h in test medium to remove exogenous estrogens.

Cell growth assays

In order to determine the effect of individual compounds on cell growth, treated cells at a density of 3000 cells/well were incubated in microtiter plates for 68 h and following 4 h with a tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). The cleavage of WST-1 to formazan by metabolically active cells was quantified by scanning the plates at 450 and 650 nm reference wavelength in a microtiter plate reader. Test medium was used as background control [16]. Three independent set of experiments performed in triplicates were evaluated. Controls with the solvents ethanol and isopropanol (0.5% v/v) were tested in parallel. Because of possible interference of natural substances with another tetrazolium salt MTT [17], we first measured the direct reductive potential of all substances tested. None of them differed from the blank (medium only) (data not shown). The growth inhibition rate was calculated as percentage of parallel untreated controls. As positive controls, TAM (10^{-5} and 10^{-4} M), GEN (10^{-5} and 10^{-4} M) and E2 (10^{-7} and 10^{-10} M) in pharmacological concentrations were used [18–20]. CR extracts were compared using the GI_{50} value, the concentration of individual compounds reducing cell growth by 50%. The GI_{50} values for each individual cell line were evaluated from the dose-response curves using Microsoft Excel computer software.

Apoptotic death assays

Morphologic assessment of apoptosis

Biocoat^{TM} culture slides (B-D, Basel, Switzerland) were used for morphological investigations of treated cells (10×10^3 well). The preapplied extracellular matrix (collagen type I) allows the *in vitro* control of cellular environments for cell growth under physiologically relevant conditions. After 24 h of culture, the chambers were removed for microscopy. Morphological analyses were performed on Giemsa stained slides and examined by oil-immersion light microscopy for occurrence of morphological changes consistent with apoptosis. The cell size scatter characteristics were investigated by flow cytometry in parallel with Annexin V adherence.

Annexin V binding analysis

Flow cytometric (FCM) analysis was performed to identify and quantify the apoptotic cells. Apoptosis was detected in cells (2×10^4) by staining with the Annexin V-FITC (Roche Diagnostics) as previously described [21]. Plasma membrane permeability was estimated by propidium iodide (PI). All the experiments were performed using FACScalibur flow cytometer (B-D, Moutain View, CA). A 5000–10,000 events per sample were collected and analyzed with the CellQuest program. Histograms of treated cells were compared to untreated controls. Vehicle treated cells (0.5% v/v ethanol, isopropanol) were always included and were not found to affect apoptosis.

M30-Apoptosense^{TM} assay

M30-Apoptosense an enzyme-linked immunosorbent assay (ELISA Kit, PEVIVA) for the quantitative measurement of the apoptosis-associated M30 neoepitope in tissue culture media, was purchased from Alexis Corp. The breast cancer cells (MCF-7, MDA-MB231) were seeded in 96 well microtiter plates at a density of 10,000 cells/well in 200 µl medium. Treatment with the CR, TAM, GEN and STP was initiated after 6 h. After 40 h, NP-40 was added to a final concentration of 0.5% and plates were frozen. By this procedure, it was possible to assay the total M30 antigen produced (cell associated + antigen released into medium). Medium/extract of 25 µl was used for the M30-Apoptosense assay performed to the instructions of manufacturer. Briefly, 25 µl standards, controls and samples were added to their assigned wells precoated with a mouse monoclonal antibody used as catcher, followed by the horse-radish peroxidase-conjugated monoclonal antibody (M30) as tracer. For the formation of the solid phase/antigen/labeled antibody sandwich the microtiter plates were agitated on shaker for 4 h at a speed of 600 rpm. After washing TNB substrate was added and the reaction was stopped after 20 min incubation (2 N H_2SO_4). The absorbance was measured in a microplate reader at 450 nm. To confirm the assay performance, the low (about 100 U/L) and high (800 U/L) controls supplied were used. The amount of M30 antigen in the samples were calculated from a standard curve and expressed as Unit per Liter (U/L).

Statistical analysis

Student's two-tailed test was employed to assess the statistical significance of difference between the untreated controls and CR-treated samples. All treatment experiments were repeated at least three times to generate statistically relevant data. GI_{50} values

were obtained by linear or exponential regression analysis of data using the Microsoft Excel software. Probability values equal to or less than 0.05 were considered significant. The comparison of histograms were evaluated statistically using the Kolmogorov–Smirnov (K-S) two samples test for overlaid histograms.

Results

Cell growth inhibitory effect of CR

To evaluate the influence of CR on the growth of mammary cancer cells, the MCF-7 cells, a well established model for the *in vitro*-investigation of estrogenic activities and MDA-MB231 (ER^-) cells were employed. Further, TAM (10^{-5} M) an ER antagonist and apoptosis inducer [18], two estrogens, E2 (10^{-10} M) that increases the speed of cell cycle and acts as antiapoptotic factor [19] and GEN (10^{-4} M) a phytoestrogen with estrogenic and antiestrogenic activity [20] were tested in parallel. Cell proliferation was measured indirectly by the cleavage of tetrazolium salt WST-1 to formazan. This assay gives an indication of the integrity of mitochondrial and also extramitochondrial NADH- and NADPH-dependent redox enzyme systems. Disturbances in these systems occur before nuclear and membrane changes are apparent in the cells. The effect of iCR was studied as a dose-response experiment for 72 h at the dilutions (5×10^{-5}–5×10^{-2}, iCR) and (10^{-5}–10^{-2}, eCR), which correspond to 0.2–200 μg/ml iCR and 0.32–325 μg/ml eCR. Both cell lines exhibited significant CR-induced suppression of growth. A dose-dependent inhibition of cell growth was observed between 20–200 μg/ml iCR and 32.5–325 μg/ml eCR (data not shown). The growth inhibitory effect of CRs was more pronounced in ER^- MDA-MB231 cells, which was mirrored in their GI_{50} concentrations (Table 1). The 50% inhibitory concentration of iCR was 54.1 ± 11.4 μg/ml (MCF-7) and 29.5 ± 3.0 μg/ml (MDA-MB231) and differed significantly ($p < 0.05$). eCR induced dose-dependent growth arrest with GI_{50} values of 80.6 ± 17.7 μg/ml in MCF-7 and 58.6 ± 12.6 μg/ml in MDA-MB231. The difference in the GI_{50} of eCR was not significant ($p = 0.105$) between MCF-7 and MDA-MB231 cells. However, the growth of both cells was inhibited almost completely by 200 μg/ml iCR and 325 μg/ml eCR. As shown in Table 1, TAM (10^{-4} M; 56.4 μg/ml) inhibited the growth of both cells almost completely while the phytoestrogen GEN (10^{-4} M, 27 μg/ml) caused about 65% of cells inhibition. At concentration of extracts of up to about 0.02 μg/ml no proliferation effect similar to that exerted by 10^{-10} M E2 was found. Survival of vehicle treated cells was 104.1 ± 1.9 and 102.5 ± 2.1% for MCF-7 and MDA-MB231, respectively.

Table 1. Influence of herbal extracts and controls on the growth of MCF-7 and MDA-MB231 breast cancer cells

	MCF-7 (ER^+)	MDA-MB231 (ER^-)
iCR (B:10720)[a]	54.1 ± 11.4	29.5 ± 3.0
eCR (B:809220)[a]	80.6 ± 17.7	58.6 ± 12.6
TAM (10^{-4} M)[b]	3.5 ± 0.7	3.5 ± 1.1
TAM (10^{-5} M)[b]	71.6 ± 4.1	58.8 ± 3.4
GEN (10^{-4} M)[b]	31.8 ± 5.3	35.9 ± 6.7
GEN (10^{-5} M)[b]	101.4 ± 9.3	87.4 ± 5.6
E2 (10^{-10} M)[b]	125.9 ± 8.7	87.0 ± 4.6

[a] GI_{50} (50% inhibitory concentration) expressed in μg/ml extract.
[b] Results expressed as percent of control growth after a 72 h incubation Means ± SD from three independent experiments are shown.

CR induced apoptosis in MCF-7 and MDA MB231 cells by activation of caspases

In principle, a reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death by either necrosis or apoptosis or a combination of these two mechanisms. We also investigated whether the extracts can induce apoptosis by monitoring the apoptosis markers: phosphatidylserine (PS) exposure on the outer leaflet of cells membrane (identified by Annexin V adherence, 22) and caspase activation, which leads to CK 18 degradation (as identified by M30 monoclonal antibody, 23). The morphological changes were inspected by microscopy and confirmed by flow cytometric characteristics of cell size and granulosity. The cells became detached from plate and became rounded already after 24 h treatment of iCR (200 μg/ml). Because loss of adhesion to the culture dishes of tumoral epithelial cells has been described as an apoptosis-related event [24], we examined the morphological apoptotic changes on Giemsa stained slides after 24 h. In opposite to good spreaded cells in the untreated controls, dose dependent loss of cells and morphological changes with cell shrinkage, chromatin aggregation were detected in the cells treated with CR

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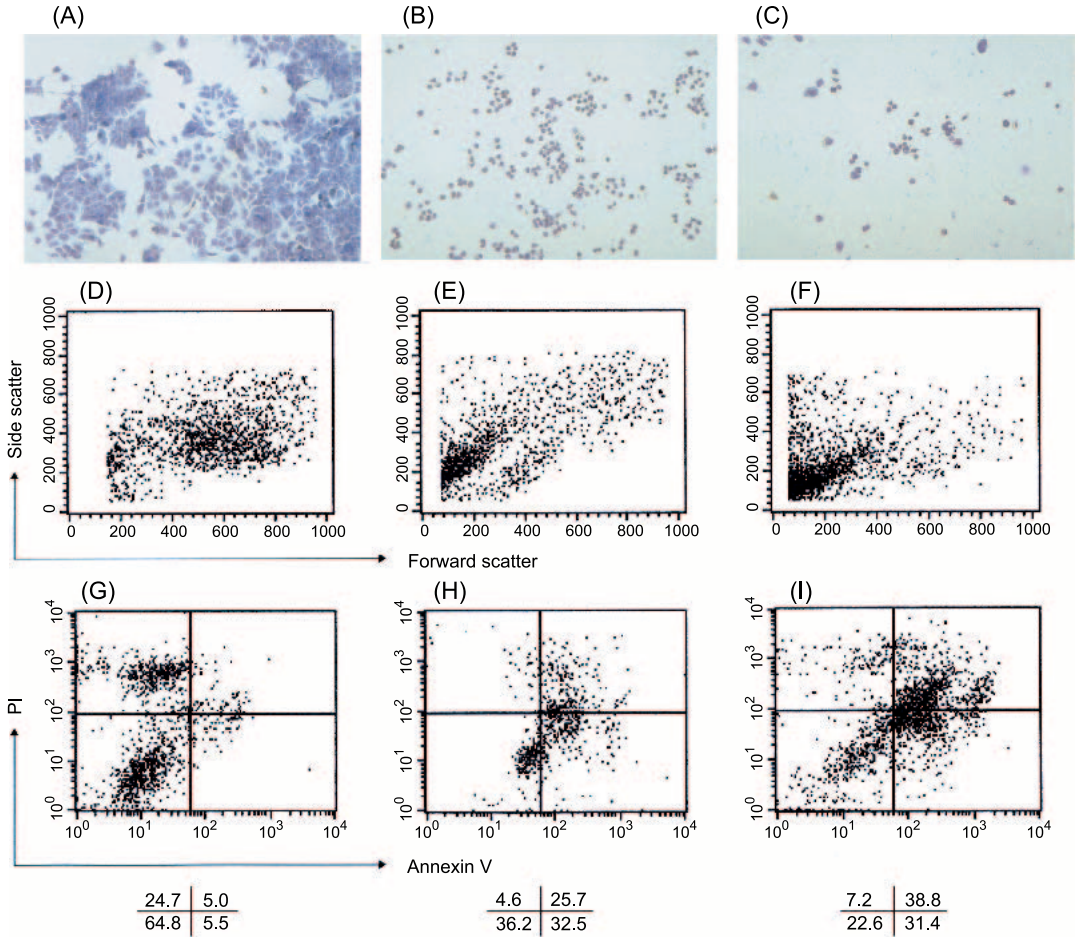

Figure 1. Morphological changes of the ER$^+$ cell line MCF-7 after 24 h treatment (A) medium only; (B) with 200 μg/ml iCR or (C) with 10 μM TAM detected by microscopy (original magnification × 100) and by light scatter characteristics using flow cytometry (cell size: forward scatter; granulosity: side scatter) after 72 h treatment (D – Ctrl; E – iCR; and F – TAM (48 h)). Apoptotic cells were simultaneously quantified by Annexin V adherence and PI staining (G – Ctrl, H – iCR; I – TAM; % of cells in the appropriate quadrants are indicated).

extract. Necrosis was less prominent than apoptosis. In Figure 1 the representative photomicrographs of MCF-7 cells after treatment with 200 μg/ml iCR in comparison to untreated control and TAM (10 μM) are shown.

To further substantiate the cell growth inhibitory effects of CR, the apoptotic cells were monitored by Annexin V adherence and plasma membrane permeability with PI uptake during 72 h treatment. In viable cells, PS is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS. Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS. The induction of apoptosis by iCR was time-and concentration-dependent (Figure 2). In ER$^-$ MDA-MB231 cells an elevation in Annexin V-positive cells up to 68.1% was found after 48 h and reached 82.6% after 72 h exposure to 200 μg/ml extract. In ER$^+$ MCF-7, there were fever apoptotic cells, iCR at concentration of 200 μg/ml inducing apoptosis in 38.5 and 54.8% after 48 and 72 h treatment, respectively. GEN at the concentration tested (50 μM) induced apoptosis in MCF-7 cells (about 45%, 72 h) in contrast to MDA-MB 231 cells, where no alteration in PS exposure compared to untreated cell was found. On the other hand, the MDA-MB 231 cell responded to 48 h of TAM (10 μM) treatment with 80% apoptotic cells in comparison to the 45% in MCF-7 cells. These data supported the additional non-genomic (non-ER-

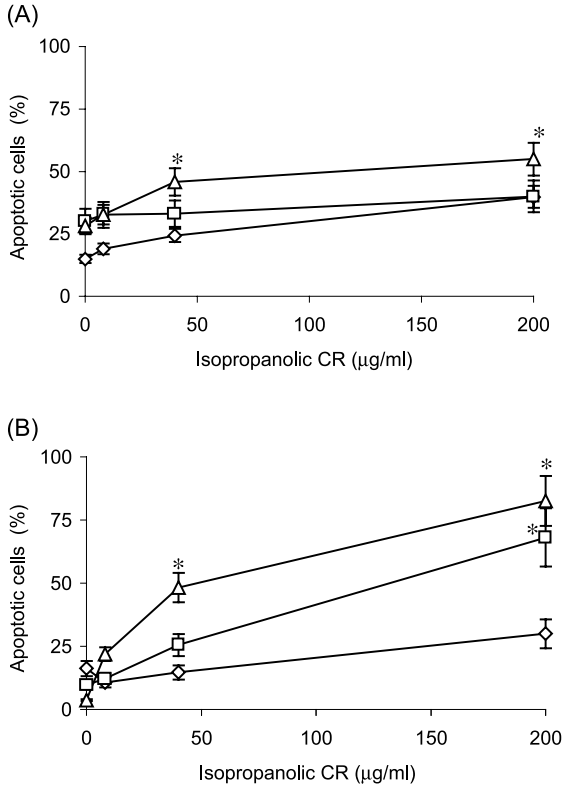

Figure 2. Apoptosis-inducing effect of iCR on MCF-7 (A) and MDA-MB231 (B) breast cancer cells expressed as percentage of Annexin V positive cells after 24 h (diamonds), 48 h (squares) and 72 h (triangles) incubation. Results are the means ± SD of three replicate experiments. *Significantly different from the untreated control at $p < 0.05$.

mediated) mechanisms of TAM-induced apoptosis. TAM induction of apoptosis in MCF-7 cells involves the ER, and requires the synthesis of new protein and mRNA. TAM induction of apoptosis in MDA-MB 231 cells depends primarily on protein synthesis [18]. The signaling proteins in the non-ER-mediated pathways include protein kinase C (PKC), transforming growth factor-β (TGF-β), calmomodulin, protooncogene c-myc, ceramide and mitogen-activated protein kinases (MAPK) [25]. The spontaneous apoptosis, lay between 14.9 and 27.3% (MCF-7) and between 10.1 and 9.8 % (MDA-MB231) after 24–72 h incubation. The solvent controls did not increase the spontaneous apoptotic rate in the two malignant cell lines tested. Apoptosis was confirmed by flow cytometry assessment of the light scattering properties of the cells. Morphological changes characteristic of apoptosis were well reflected by reduced ability to scatter light in the forward direction (FSC) and an increase or no change in the 90° light scatter (SSC). As representative for the treated cells the microscopic and flow cytometric investigations either scatter characteristics also two parameters histograms (Annexin V/PI) on MCF-7 cells are shown in Figure 1. As controls untreated and of 48 h TAM (10^{-5} M) treated cells were used. eCR induced apoptosis at comparable concentrations to iCR in both breast carcinoma cells (data not shown).

During apoptosis, caspases cleave various cellular proteins. In epithelial cells, one of those proteins is the intermediate filament CK18. The M30 antibody recognizes a neo-epitope in the C-terminal domain of CK18 (amino acids 387–396) which is exposed after cleavage. Several caspases appear to be capable of cleaving CK18, therefore an increased M30 activity after induction of apoptosis suggests a caspase involvement in drug-induced cell apoptosis [26, 27]. Involvement of caspases activation in CR extract-induced apoptosis were investigated by M30 activity on treated cells and is presented in Figure 3. All cells were treated for 40 h and STP (0.5, 1.0 and 2.0 μM) was used as positive control. Both, iCR and eCR exerted comparable increase in the total M30 activity and confirmed the results obtained by Annexin V-binding evaluated by FCM. The greatest increases compared to the uninduced controls in the total M30 antigen (cell associated + antigen released into medium) activity were detected at concentrations of 65 and 40 μg/ml of eCR and iCR extract, in MCF-7 (4–4.5 fold), respectively. The effect of CR extracts was comparable to that of STP (4.1-fold) in the ER^+ MCF-7 cells and superior to TAM (2.8-fold). The greatest M30-antigen level (4397 U/L) was found by the treatment of MCF-7 cells with 40 μg/ml iCR. Increasing the concentration of iCR did not cause further elevation of M30-antigen level. M30 activity in cell extracts and culture supernatants from MDA-MB231 cells increased by 90%. As shown in Figure 3(B), total caspase-cleaved CK18 levels of 2515 U/L (M30) were observed in 0.5 μM STP-treated cultures, an increase of 21-fold over untreated ER^- MDA-MB231 cells. Both cells responded equally to TAM treatment with about 2.8-fold increase relative to uninduced controls. However, no induction in the M30 antibody level was detected after 40 h treatment of both breast cancer cells with 50 μM GEN. Accumulation of M30-antigen was higher in the media of untreated MCF-7 (975 U/L) compared to MDA-MB231 (119 U/L) due to basal apoptosis in the estrogen-free culture condition.

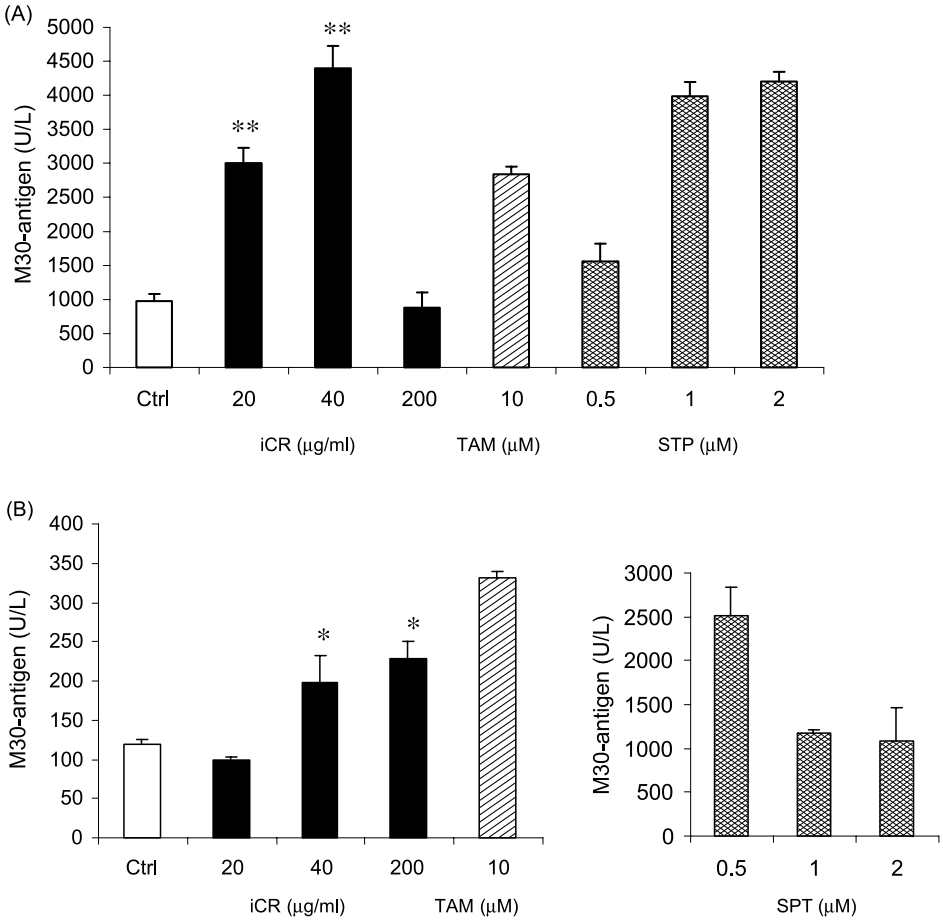

Figure 3. Induction of total CK18 cleavage in cell extracts and tissue culture supernatants in MCF-7 (A) and MDA-MB231 (B) cells. Cells (10×10^3) were treated for 40 h with indicated concentrations of iCR, TAM and STP. M30-activity was measured with ELISA and the values are expressed as U/L. Bars represents means ± SD from two experiments ($^* p < 0.05$; $^{**} p < 0.01$).

Discussion

Clinically, CR extract is used to alleviate perimenopausal and postmenopausal symptoms. Like many other herbal products, CR extract is a multicomponent mixture with pharmacologically active substances. The biologically active moieties of black cohosh are attributed to a number of triterpene glycosides (e.g., actein, 27-deoxyactein, cimicifugoside, cimiracemosides) and phenolic compounds such as fukinolic acid or cimicifugic acids A, B, E and F [28, 29]. Estrogenic activity (proliferation of ER^+ MCF-7 cells *in vitro*) was postulated for some individual monosubstances of CR [5]. In contrast, growth of ER^+ cell lines MCF-7 and T47D was inhibited by whole CR extract [7, 9, 30]. In addition the effect of E2 on proliferation and gene expression in estrogen-inducible MVLN cells was antagonized by the whole extract of CR [7]. Therefore the aim of the present study was to substantiate the inhibitory effect of CR on both ER^+ and ER^- breast carcinoma cells and to investigate the mode of cell death, namely apoptosis.

We first established the 50% cell inhibitory concentration using WST-1 assay. The ER^- cells were significantly more sensitive than ER^+ cells with an approximately 2-fold variation in the GI_{50} concentration of the CR extracts (Table 1). Both extracts (iCR and eCR) exerted comparable effects. GI_{50} values established by us were about 10-fold higher than the 50% inhibitory values described by others [7, 9] due to the different systems used for estimation. We measured the antiproliferative activity indirectly by reduction of tetrazolium salt by cellular enzymes of viable cells. WST-1 is reduced by NADH- and NADPH-dependent

redox enzyme systems. Other investigators [7, 9] employed the incorporation of [^{3}H] thymidine into the DNA of dividing cells. This method seems to be more sensitive than the WST-1 assay. However, we could clearly document the dose-dependent antiproliferative activity of CR on breast tumor cells. The antiproliferative action of CR in breast cancer cells is probably evoked by genomic (ER-mediated) [7] and non-ER-mediated mechanisms because of the various physico-chemical properties of individual components of CR. The down-regulation of ER-α mRNA by two abundant flavonoid phytoestrogens genistein and quercetin was found to be responsible for the growth inhibition of MCF-7 cells but not for their cytotoxic effect [31]. Discovery of active compounds from natural products with cytocidal (e.g., apoptosis-inducing) rather than cytotoxic ability is of great interest for cancer treatment. Screening for anti-cancer substances is commonly conducted using viability assays. An inherent problem with this approach is that all compounds that are toxic and growth inhibitory, irrespective of their concentration-dependent mechanism of action, will score positive. Apoptosis is essential for normal physiological development but is also critical in eliminating any abnormal cells after exposure to genotoxic or DNA-damaging agents. Therefore, in order to assess the role of apoptosis in the growth inhibition of mammary cancer cells by CR treatment we employed the Annexin V assay, detected a neo-epitope on CK18 exposed after cleavage by caspases using M30 antibody and microscopically inspected dying cells. The morphological changes occurred early, after 24 h treatment, with the loss of adhesion and number of the cells (Figure 1). The apoptotic changes characterized by PS exposure (detected by Annexin V adherence) and plasma membrane permeabilty (using PI) followed later (after 72 h), as shown in Figure 1 on MCF-7 cells are representative for both cell lines. Simultaneously, the changes in light scatter characteristics of cell size confirmed apoptosis. As secondary necrotic cells bind both Annexin V and PI, and since PI staining indicates final necrosis but does not exclude cell death through apoptosis, the kinetics of cell death by CR (24–72 h treatment) were studied (Figure 2). The spontaneous apoptotic rate of MCF-7 cell in comparison to MDA-MB231 was higher (up to 30% at 72 h), probably due to the estrogen deprived test condition. About 50% ER^+ cells and about 80% ER^- cells bound Annexin V after 72 h treatment with 200 μg/ml iCR thus indicating apoptotic alterations. These findings correspond with the results from the proliferation assays and suggest that CR cytotoxicity appears to be explained in part by the induction of apoptosis.

Further, we chose the M30 antibody for confirming the apoptosis induced by CR. The increased activity of M30 antibody in epithelial cells correlates with the caspases-activation during apoptosis [15, 23, 27]. The MCF-7 cells in spite of their caspase-3 deficiency [32] showed a high responsiveness to the CR treatment, comparable with STP a potent non-selective protein kinases inhibitor [33]. The deficiency of caspase-3 are responsible for the relative insensitivity of MCF-7 cancer cells to many chemotherapeutic agents. Effector caspases distinct from caspase-3, such as caspase-7, cleave CK18 at the C-terminal site (aa396) [23], generating M30-antigen. Many of the present breast cancer chemopreventive and chemotherapeutic agents can have undesirable side effects. Therefore, there has been interest in the use of natural alternative substances to prevent or treat breast cancer without the side effects. The apoptosis-inducing action of complex herb extract (CR) reported here also in estrogen-related cancer cells must be mediated through mechanisms other than its weak binding to estrogen receptors. In the media from untreated MCF-7 cells the accumulation of M30 antigen was observed to be related to their spontaneous apoptosis, which is in accordance with our findings using Annexin V adherence. In comparison to MCF-7 cells, only a 90% increase in the M30 activity was found in MDA-MB231 cells. Because CK18 cleavage by caspase activation occurs mainly in the early phase of apoptosis, it is possible that the 40 h incubation time applied here was too long for MDA-MB231 cells which were already in the late phase of apoaptosis. This hypothesis is supported by their response to various concentrations of STP. The lowest concentration (0.5 μM) of STP induced a 21-fold elevation compared to uninduced control. However, a further increase in the concentration of STP did not increase the activity of M30, which could be explained by the dose- and time-dependent apoptosis (Figure 3). An another reason for the lower cleavage of CK18 associated with apoptosis induction could be the expression of the mutant p53 gene in the highly invasive MDA-MB 231 cells in contrast to the wild-type p53 expressing minimally invasive MCF-7 cells, as reported for GEN [34]. No alteration in the level of M30 antibody after 40 h treatment with GEN in either breast cancer cell line was detected. Our present findings are in accordance with the reported ability of GEN which arrested cells in the G2/M stage of the cell cycle [21, 35], but

the apoptotic signs could only be detected after 4–6 days of incubation with 50 μM GEN in ER^+ MCF-7 and MDA-MB468 cells [34, 35]. In the present study we provide evidence that CR extract induced apoptosis at a comparable or higher degree than that of the estrogen antagonist TAM and that its effect is superior to that of phytoestrogen GEN, an abundant compound of soy and red clover extracts [6]. Taken together the fact, that ER^- cells were more sensitive to growth inhibition by CR than ER^+ cells and that a higher caspase activity was induced in ER^+ cells, could indicate that apoptosis is not necessarily the mechanism behind growth inhibition.

Breast cancer is the most common malignancy affecting women, with more than one million cases occurring worldwide annually as recently reported by WHO [36]. To relieve the menopausal symptoms, HRT therapy has been used to treat the resulting estrogen deficiency symptoms. Because of the evidence of a link between breast cancer and postmenopausal HRT, there is a difficult therapeutic problem presented by women with a history of breast cancer whose symptoms of hormone deficiency are so severe that it becomes appropriate to consider treatment with estrogen. Our present results firstly showed the apoptosis-inducing properties of CR extract in both ER^+ MCF-7 and ER^- MDA-MB231 breast cells *in vitro* by activating of caspases. Because of the absence of a systemic estrogenic effect in *in vivo* studies [8, 28, 37] the CR extract could be a safe and effective alternative to estrogen therapy in a therapeutic recommended dosages. Further *in vitro* and *in vivo* experiments are necessary to exploit its apoptosis-inducing mechanisms and for its possible beneficial health effect.

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