Report

Purging of contaminating breast cancer cells from hematopoietic progenitor cell preparations using activation enhanced cell death

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Summary

Activation enhanced cell death (AECD) involves stimulating cells with growth or activation signals while concurrently blocking calcium influx. In this study, we have evaluated the effect of AECD on human breast cancer cells. MCF-7 or MDA-MB-231 cells treated with Ca²⁺ influx blockers econazole or ketotifen for 24 h underwent a dose-dependent, irreversible loss of viability, and clonogenicity. Two-hour treatment of these cells with higher concentrations of the drugs also resulted in loss of clonogenicity, but morphological indicators of cell death were apparent only after longer incubation. Loss of clonogenicity could be enhanced almost 10-fold by co-stimulation of the cells with the agonists EGF or bombesin. Econazole was also effective in inducing cell death in multi-drug resistant MCF-7_{adr} cells. Human hemopoietic progenitor cell sensitivity to econazole or ketotifen was evaluated by colony assay. Under conditions resulting in 2.5–3 logs of breast cancer cell loss, 60–70% of hemopoietic progenitors could be recovered. We further evaluated the effect of econazole on breast cancer cells present in mobilized hemopoietic cells obtained from patients undergoing high dose chemotherapy with autologous stem cell support. In six of eight samples evaluated, cytokeratin-positive breast cancer cells could be detected by immunofluorescence microscopy and colony formation. Breast cancer colonies were reduced 60–500-fold or more after exposure to econazole while hemopoietic colonies were typically reduced only 2-fold. In all cases, addition of EGF as an activator either had no evaluable effect or enhanced breast cancer cell loss. We conclude that Ca²⁺ influx blockade with concurrent EGF stimulation is a promising approach for purging breast cancer cells from hemopoietic progenitor cell preparations.

Abbreviations: PBSC: peripheral blood stem cell; ABMT: autologous bone marrow transplantation; AECD: activation enhanced cell death; EGF: epithelial growth factor; CFU-GM: colony formation unit-granulocyte and macrophage; BFU-E: erythroid burst forming unit

Introduction

The use of high dose chemotherapy with autologous stem cell support (ASCT) as a strategy to improve the outcome for women with advanced breast cancer continues to be an active area of clinical research [1–6]. However, using a variety of immunohistochemical or cell culture techniques, a number of investigators have reported that clonogenic breast cancer cells can be detected in bone marrow or peripheral blood

stem cells from patients with metastatic or high-risk, node-positive breast cancer [7–10]. Although the detection of such cells may be simply an indicator of a larger burden of disease, it is possible that the re-infusion of such cells may contribute to relapse, as has been elegantly demonstrated in other tumor types [11–13]. These observations have led to the development of *ex-vivo* approaches for removing contaminating tumor cells, or purging, of stem cell products. A number of approaches including the use of che-

motherapeutic agents (4-hydroxycyclophosphamide) [14–17] and positive enrichment of hemopoietic progenitor cells using CD34⁺ immunoseparation, have been attempted with variable success [18–20]. More effective methods of purging hematopoietic stem cell products are an important part of the ongoing research to determine the place of ASCT in the treatment of breast cancer and, would also be of relevance for the treatment of other cancers in which high dose chemotherapy is a treatment option.

Recently, we identified a novel mechanism for inducing cell death [21]. This mechanism, which we refer to as activation enhanced cell death (AECD), involves stimulating cells with growth or activation signals while concurrently blocking calcium influx. Our initial experiments demonstrated that concurrent blockade of Ca²⁺ influx while stimulating mast cells or leukemic cells with the growth factor stem cell factor (SCF) could reduce clonogenicity by up to 3 orders of magnitude. Our experiments also showed that the most highly activated cells were the most sensitive to cell death induction, that phospholipase C activation likely contributed to cell death, and that bcl-2 overexpression was ineffective in protecting cells from death. We further demonstrated that constitutively activated cells such as p815 mastocytoma cells were sensitive to Ca²⁺ influx blockers alone, likely due to their high degree of autonomous stimulation.

Many studies have shown that breast cancer cells can overexpress growth factor receptors such as the EGF receptor, ErbB2 (Her2/neu), a receptor tyrosine kinase closely related to the EGF receptor, and fibroblast growth factor (FGF) receptor [22–25]. Autocrine stimulation by growth factors has been demonstrated in some instances [26]. We hypothesized that in these instances, the autonomous stimulation of these cells might render them sensitive to cell death by AECD. Here we show that cell death can be induced in the human breast cancer cell lines MCF-7 or MDA-MB-231 through exposure to Ca²⁺ influx blockers. We also show that in some circumstances, this cell death can be enhanced by concurrent stimulation with EGF or bombesin. We also demonstrate, using samples from patients undergoing high dose chemotherapy with autologous hemopoietic stem cell support, that human hemopoietic progenitor cells are considerably less sensitive to AECD than breast cancer cells suggesting that this approach may be effective in *in vitro* purging.

Materials and methods

Human breast cancer cell lines and culture conditions

MCF-7 and MDA-MB-231 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). MCF-7_{adr} cells were obtained from Dr Ian Tannock (Toronto, Canada). The cell lines were grown routinely as monolayer culture in Dulbecco's minimal essential media (DMEM) supplemented with L-glutamine (2 mM), penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, GIBCO) in an atmosphere of 5% CO₂ at 37°C. MCF-7 cultures were also supplemented with bovine insulin (10 µg/ml, GIBCO). The cell lines were passaged weekly. Econazole and ketotifen were purchased from Sigma Chemical Co. (St. Louis, MO). These compounds were freshly dissolved in DMSO before use and added to the culture media at the indicated concentrations, the final concentration of solvent DMSO was always less than 0.1%. Epidermal growth factor (EGF) was obtained from R & D Systems (Minneapolis, MN, USA). Bombesin is the product of Sigma Co.

Bone marrow and PBSC samples

Normal bone marrow specimens were obtained from healthy individuals serving as allogeneic bone marrow transplantation donors. Samples of mobilized peripheral blood stem cells (PBSCs) from eight women with breast cancer (two high risk primary, and six metastatic) were obtained from patients in accordance with local Research Ethics Board Approval and following informed consent from the patients. Before apheresis, patients received chemotherapy and granulocyte colony-stimulating factor (G-CSF) to mobilize progenitor cells. The mononuclear cell (MNC) fraction was separated by density gradient centrifugation. Briefly, bone marrow or PBSC was diluted with Iscove's modified DMEM (IMDM), layered on Ficoll-Hypaque (Pharmacia, Sweden), centrifuged at 2000 rpm for 20 min, and washed twice with media.

Cell viability and proliferation

Log phase MCF-7 and MDA-MB-231 cells were trypsinized, washed and exposed to different concentrations of econazole and ketotifen for 24 h with or without growth factors. Both adherent and non-adherent cells were harvested and stained with Trypan Blue. Cell viability was determined by dye exclusion. To test the antiproliferative effect of these drugs,

The XTT assay was used to measure the cell proliferation as described previously [27]. Briefly, 4,000 treated and untreated cells were plated in 96-well microtiter plates in 100 μ l volume of media and allowed to grow for 48 h. Fifty microliter of XTT reagent (333 μ g/ml) and phenazine methosulfate (2.55 μ g/ml, electron coupling reagent) were added to each well. The absorbance was read on a microtiter plate reader (Molecular Device Ltd., Crawley, West Essex, UK) using a wavelength of 490 nm after 2–4 h incubation at 37°C.

Breast cancer clonogenic assay

For the breast cancer clonogenic assay, MCF-7 and MDA-MB-231 cells were mixed with bone marrow mononuclear cells in a ratio of 1:100 (tumor:MNC). Cell mixtures were treated with different drugs as described above for cell viability. Both adherent and non-adherent cells were collected and washed with fresh media. Cell aliquots (5×10^3 /ml tumor cell, or 5×10^5 /ml mixed cell populations) were plated in 1 ml of 0.3% agar over 1 ml of underlayer of 0.5% agar prepared in IMDM containing 10% horse serum (GIBCO). The upper layer consisted of 20% FBS, 10 μ g/ml of bovine insulin, 2.5 μ g/ml of hydrocortisone, 5×10^{-7} M of 17 β -estradiol (Sigma) and 50 ng/ml of EGF(R & D system). Colonies larger than 50 μ m in size were scored after 14 days of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air. To culture breast cancer colonies from patient PBSC samples, culture conditions similar to that for the cell lines was used [7]. Briefly, 1×10^6 mononuclear cells were seeded into 35 mm petri dishes in a culture system consisting of 20% of FBS in IMDM, 0.3% agar supplemented with 5 μ g/ml EGF, 0.1 μ g/ml human recombinant insulin-like growth factor I (IGF-1) and 50 ng/ml of human recombinant GM-CSF. At least 10 dishes per sample were plated for each group. Colonies were counted after 14 days incubation in humidified chamber at 37°C and 5% CO₂. The breast cancer colonies could be easily distinguished from CFU-GM colonies by morphology. In some cases, the origin of the breast cancer colonies was confirmed by immunostaining of cells recovered from the agar culture.

Hemopoietic progenitor colony formation

Hemopoietic progenitor cell viability was determined by colony formation in methylcellulose. Cells (2×10^5 /ml for bone marrow, 4×10^5 /ml for PBSC)

were collected, washed and plated in triplicate into 1 ml of Methocult (Stem Cell Technologies, Vancouver, Canada) consisting of 0.9% methylcellulose in IMDM, human recombinant GM-CSF (20 ng/ml), erythropoietin (2 U/ml), stem cell factor (50 ng/ml), and interleukin-3 (10 ng/ml). CFU-GM and BFU-E were counted after 14 days of incubation at 37°C in 5% CO₂.

Immunofluorescent detection of contaminating breast cancer cells in PBSC

For detection of breast cancer cells in PBSC samples, standard indirect immunofluorescence was used. Isolated mononuclear cells were attached onto slides by cytospin (Shandon, UK). 5×10^5 cells/slide and a total of 10 slides were made for each sample. Slides were air-dried, fixed in 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton-X 100 for 15 min and blocked with 3% BSA in PBS for 15 min. For immunostaining, anticytokeratin antibodies AE1/AE3 (Boehringer Mannheim) and fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibody (Amshersham Pharmacia Biotech) were applied consecutively. Positive (MCF-7) as well as negative (IgG1 isotype) samples were included in each staining process. At least 3×10^6 cells (six slides) were examined for the presence of contaminating tumor cells by epifluorescent microscopy (Leica).

Hoechst 33258 dye staining

The presence of nuclear morphology characteristic of apoptosis was evaluated by staining with Hoechst 33258 staining. MCF-7 or MDA-MB-231 cells were collected, washed with PBS, fixed in 5% formalin overnight and washed twice with PBS. The fluorescent dye Hoechst 33258 (4 μ g/ml) was added to the suspension and stained for 15 min, the cells were dropped onto slides, coverslipped, mounted with glycerol, and evaluated by epifluorescent microscopy.

DNA fragmentation

After treatment, 6×10^6 MCF-7 or MDA-MB-231 cells were lysed in buffer containing 20 mM EDTA, 0.5% (v/v) Triton X-100, 10 mM Tri-Hcl (pH8.0), and proteinase K (0.4 mg/ml) for 1 h at 55°C. Cell lysates were centrifuged for 15 min at $13,000 \times g$. The aqueous phase was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) as reported previously [28]. DNA was precipitated with

0.3 M sodium acetate and two volume of cold 100% ethanol and then incubated with RNase (20 µg/ml) overnight at 37°C. DNA samples were electrophoresed through a 1.8% agarose gel at 80 V for 2.5 h.

Statistical analysis

Colony data in the bar graph was analysed by unpaired *t* test. All other data were analysed by two-way analysis of variance, with differences between individual means determined by Bonferroni's post-tests. Data were expressed as means ± SEM.

Results

Econazole or ketotifen induce breast cancer cell death

Our previous work demonstrated that mast cells and leukemia cells could undergo cell death when stimulated with the growth factor SCF while concurrently blocking Ca^{2+} influx [21]. Since many breast cancer cells express high levels of Ca^{2+} -mobilizing growth factor receptors, this suggested to us that breast cancer cells might also be sensitive to Ca^{2+} influx blockade in an activation-dependent manner. We, therefore, tested the effects of the Ca^{2+} channel blockers econazole and ketotifen on cell viability and clonogenicity of human breast cancer cells. These compounds are known to target the store-operated Ca^{2+} channel that is activated in response to the mobilization of Ca^{2+} from intracellular stores located in the endoplasmic reticulum [29]. MCF-7 or MDA-MB-231 cells were exposed to increasing concentrations of econazole or ketotifen for 24 h. The cells were collected and assayed for viability by Trypan Blue dye exclusion. As shown in Figures 1(A) and (C), both drugs cause loss of cell viability in a dose-dependent manner. The concentrations required to induce cell death are slightly higher in magnitude than those required to kill bone marrow-derived murine mast cells or 32D-Kit leukemia cells [21], possibly due to species-based differences in sensitivity. We also evaluated the effects of these drugs on clonogenicity. As shown in Figure 1(B) and (D), both econazole and ketotifen cause a sharp loss of cell clonogenicity above 10 and 150 µM, respectively. This sharp dose dependence for colony formation is also similar to that observed previously with 32D-Kit leukemia cells. Treatment with econazole was particularly effective. At the highest dose tested (15 µM),

colony formation was reduced to below the threshold of detection for the assay.

Morphological characteristics of econazole or ketotifen-induced cell death

Our previous work demonstrated that exposure of mast cells, p815 mastocytoma cells or 32D-Kit leukemia cells to econazole or ketotifen resulted in morphological characteristics associated with apoptosis including membrane blebbing, DNA fragmentation, and cleavage of caspase substrates. We, therefore, examined the morphological consequences of overnight incubation with these compounds on breast cancer cells. As shown in Figures 2(A) and (B), microscopic examination of MCF-7 and MDA-MB-231 cells incubated in econazole or ketotifen showed a characteristic change in cell morphology. Treated cells detached from the flask and became pyknotic (Figure 2(A)). Using the DNA staining fluorescent dye Hoechst 33258, we found that significant chromatin condensation occurred in both cell lines but only infrequent nuclear segmentation was observed (Figure 2(B)). Furthermore, no DNA laddering was seen in econazole or ketotifen treated MCF-7 and MDA-MB-231 cells (Figure (3)). We conclude that unlike mast cells, p815 cells or 32D-kit leukemia cells [21], these breast cancer cells do not exhibit classical apoptotic morphology.

Enhancement of cell death with EGF or bombesin

Although both MCF-7 and MDA-MB-231 cells are growth autonomous in standard cell culture, these cells have also been reported to respond to EGF [30, 31] and bombesin [32, 33]. We, therefore, investigated the effect of co-stimulating these cells with these activating factors in combination with the Ca^{2+} influx blockers. For treatment of MCF-7 cells with econazole (12 µM, 24 h), co-stimulation with EGF or bombesin enhanced clonogenic log reduction from 2.3 to 2.7 and 3.0, respectively (Figure 4(A)). A similar synergy was also observed for MDA-MB-231 cells. Stimulation with EGF or bombesin enhanced clonogenic log reduction from 2.1 to 2.6 and 3.1, respectively (Figure 4(A)). For cells treated with ketotifen, bombesin, but not EGF, enhanced the efficacy of ketotifen against MCF-7, whereas only EGF has some influence on the ketotifen induced cell death of MDA-MB-231 cells (Figure 4(B)). Importantly, the addition of these growth factors did not protect the breast cancer cells

Figure 1. Effect of econazole and ketotifen on MCF-7 and MDA-MB-231 cells. Cells were incubated with the indicated concentrations of econazole or ketotifen for 24 h in the presence of 0.5% FBS. Both floating and adherent cells were collected and cell viability was determined by dye exclusion ((A), (C)). For clonogenicity assays, MCF-7 or MDA-MB-231 cells was mixed with normal bone marrow mononuclear cells in a ratio of 1:100 (tumor: MNC), 5×10^5 treated or untreated cells were plated into petri dishes using a double-layer agar culture system ((B), (D)). Results represent the mean values of triplicate determinations. (A), (B): Econazole. (C), (D): Ketotifen.

from the influx blockers under any of the conditions tested.

Effect of econazole and ketotifen on hemopoietic progenitor cells

Toxicity to normal hemopoietic stem cells has limited the use of cytotoxic agents for *in vitro* purging. Therefore, we also investigated the effect of econazole and ketotifen on human hemopoietic progenitor cells. Bone marrow mononuclear cells obtained from normal donors were treated with econazole or ketotifen for 24 h and then plated in methylcellulose in the presence of hemopoietic growth factors. After 14 days, the plates were scored for colony growth. As shown in Figure 5, both econazole and ketotifen

caused a reduction in colony formation, but the degree of clonogenicity loss was much lower than that observed for breast cancer cells. For example, 12 μM econazole, which reduced breast cancer clonogenicity by 2–3 orders of magnitude, caused only a 69% reduction of CFU-GM (Figure 5(A)). Similarly, 200 μM ketotifen, which reduced breast cancer clonogenicity by 2–2.5 orders of magnitude, causes only a 68% reduction in CFU-GMs (Figure 5(B)). BFU-Es appeared to be somewhat more sensitive to econazole or ketotifen treatment. For example, 12 μM econazole reduced BFU-E levels by 90%, while 200 μM ketotifen reduced BFU-E levels by 95%. In both cases, addition of EGF or bombesin to these cells had no additional effect on hemopoietic colony-forming ability.

Figure 2. Microscopic analysis of cells treated with econazole or ketotifen for 24 h. (A) Inverted microscopic morphology. (B) Hoechst stain of cells.

Efficacy of 2-h treatments

Human hemopoietic progenitor cells are surprisingly robust and can be maintained for several hours in the absence of supporting growth factors. However, some loss of cell viability is possible during the 24-h incubation period used in the experiments described above. In order to minimize possible loss of cells during this period, we investigated the effectiveness of shortened

treatment periods. MCF-7 and MDA-MB-231 cells were exposed to econazole in serum-free medium for 2 h at 37°C. The cells were washed with fresh media, and then evaluated for viability. Viability was determined by culturing the cells in fresh media for 48 h following treatment and then measuring the ability of the cells to metabolize XTT. As shown in Figures 6(A) and (B), both MCF-7 cells and MDA-MB-231 cells lost viability when exposed to econazole for 2 h. The

Figure 3. DNA ladder analysis of treated or untreated MCF-7 and MDA-MB-231 cells. M: marker, 1 kb + 100 bp DNA ladder. Lane 1: Negative control (untreated U937 cells). Lane 2: Positive control (U937 cells treated with 10 μ M econazole). Lane 3–5: MCF-7 cells, vector (lane 3), econazole 12 μ M (lane 4), ketotifen 200 μ M (lane 5). Lane 6–8: MDA-MB-231 cells, vector (lane 6), econazole 12 μ M (lane 7), ketotifen 200 μ M (lane 8).

concentrations required for inducing cell death were somewhat higher than those needed for the 24-h incubation period. Fifteen micromol econazole appeared to be very effective in eliminating cell viability. We also observed that ketotifen was much less effective over the 2-h treatment period than econazole (Zhang and Berger; data not shown).

We also compared the ability of 2-h treatment with econazole to reduce clonogenicity of breast cancer cells and hemopoietic progenitor cells. MCF-7 or MDA-MB-231 cells were mixed with normal human bone marrow mononuclear cells and treated with 15 μ M econazole for 2 h. The cells were then washed and plated in conditions for either breast cancer colony formation or hemopoietic colony formation. As shown in Figure 6(C), MCF-7 and MDA-MB-231 clonogenicity was reduced by 2.7 and 2.6 logs, respectively. In both cases, addition of EGF or bombesin enhanced log reduction. The combination of 15 μ M econazole plus EGF resulted in MCF-7 and MDA-MB-231 log reductions of 3.1 and 3.3, respectively. Furthermore, these conditions resulted in only a 30% reduction of CFU-GMs and 39% reduction of BFU-E (Figure 6(D)). Therefore, 2-h treatment times appear to be as effective in reducing breast cancer viability as

24 h incubations. This treatment protocol also displays activation enhancement, particularly with EGF, and demonstrates decreased toxicity to normal human hemopoietic progenitor cells, particularly BFU-E.

Doxorubicin-resistant MCF-7 cells are sensitive to econazole

One of the most common mechanisms by which tumor cells become resistant to chemotherapy is from the overexpression of P glycoprotein and related proteins [34, 35]. These plasma membrane-associated proteins promote the efflux of lipophilic drugs thus decreasing their effectiveness. Since econazole is a lipophilic compound, we wished to determine whether this compound was susceptible to the multi-drug resistance phenotype. We, therefore, evaluated the efficacy of econazole in inducing cell death in MCF-7_{adr} cells. These cells overexpress P glycoprotein and display a multi-drug resistant phenotype, including resistance to doxorubicin. Both parental MCF-7 and MCF-7_{adr} cells were exposed to either doxorubicin or econazole plus EGF for 2 h and then assayed for clonogenicity. As shown in Figure 7(A), MCF-7_{adr} cells display remarkable resistance to doxorubicin. At both 1 and

Figure 4. Enhancement of breast cancer cell depletion using co-stimulation with agonists. MCF-7 or MDA-MB-231 cells was exposed to econazole or ketotifen for 24 h in the presence or absence of growth factor, treated cells were seeded for tumor colony formation using the same culture conditions specified in Figure 1. (A) Econazole 12 μ M, EGF 100 ng/ml, bombesin 1 μ M. (B) Ketotifen 200 μ M. The differences of all the treatments versus untreated control are significant ($p < 0.001$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to econazole or ketotifen alone).

10 μ M doxorubicin, no MCF-7 colonies could be detected, consistent with clonogenic reduction of over 3 orders of magnitude whereas MCF-7_{adr} colony formation was reduced by only 30% at these concentrations. In contrast, we observed that MCF-7_{adr} cells displayed enhanced toxicity to econazole plus EGF compared to MCF-7 cells. At 15 μ M econazole, no MCF_{adr} colonies could be detected, consistent with a log reduction of at least 3.3 (Figure 7(B)). These results, therefore, indicate that econazole is not subject to multi-drug resistance by P glycoprotein overexpression.

Purging breast cancer cells from mobilized hemopoietic progenitor cell preparations

Our observations indicate that AECD, and in particular econazole plus EGF, displays considerable differential effectiveness in inducing cell death in breast cancer cell lines compared to hemopoietic progenitor cells. These results, therefore, suggest that econazole

can be used to purge hemopoietic progenitor cell preparations of possible contaminating breast cancer cells. We, therefore, tested this possibility on mobilized hemopoietic progenitor cell preparations obtained from eight patients undergoing high dose chemotherapy with stem cell support. The samples were evaluated for breast cancer cell content by immunofluorescence with cytokeratin-specific antibodies and by colony formation in soft agar (Figure 8). As summarized in Tables 1 and 2, six of eight patient samples were found to contain cytokeratin-positive cells by immunofluorescence. The frequency of cytokeratin-positive cells ranged from 0.2 to 27.5 (mean 9.4) per 10^6 mononuclear cells. The same six samples were also found to contain cells capable of forming colonies in soft agar. In some cases, breast cancer colonies were removed from the agar culture and confirmed to be cytokeratin positive by immunofluorescence (not shown). The frequency ranged from 3 to 17.7 (mean 13.7) per 10^6 mononuclear cells. Although the pres-

Figure 5. Recovery of bone marrow CFU-GM and BFU-E progenitors following treatment of econazole or ketoconazole. Mononuclear cells from normal bone marrow donors were separated and incubated with drugs for 24 h in 0.5% of FBS, 2×10^5 treated cells were washed with fresh media and grown for hematopoietic colony formation using Methocult supplemented with GM-CSF (20 ng/ml), IL-3 (10 ng/ml), stem cell factor (SCF, 50 ng/ml) and erythropoietin (2 U/ml). (A) and (B): Dose response of CFU-GM and BFU-E to econazole (A) and ketoconazole (B). (C) and (D): Influence of combination of SOC inhibitor with growth factors on the hematopoietic recovery. (C): Econazole 12 μ M. (D): Ketoconazole 200 μ M. Growth factors used are EGF (100 ng/ml), bombesin (1 μ M).

Figure 6. Efficiency of 2h treatment of SOC inhibitor to breast cancer cells or bone marrow mononuclear cells. Mixture of tumor and MNC were treated with econazole (15 μM) or econazole plus EGF for 2 h in the absence of FBS, cells were resuspended in fresh media and cultured for breast cancer colony or CFU-GM, BFU-E. For cell viability, MCF-7 or MDA-MB-231 cells were incubated with different concentration of econazole for 2 h, 4000 cells were collected and cultured in 96-well plate for another 48 h before measured for XTT density (details are described in Materials and methods). (A) and (B): XTT metabolism (A, MCF-7, B, MDA-MB-231). Values are percentage of control. (C) and (D): Clonogenic recovery ((C), breast cancer cells, (D), hematopoietic progenitors). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (compared to econazole alone).

Figure 7. Effect of doxorubicin or econazole on parental and multi-drug resistant MCF-7 cells. Cells were incubated for 2 h in serum free conditions, washed and plated for colony formation in soft agar. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

ence of cytokeratin-positive cells and colony-forming cells corresponded, the frequencies did not. This may reflect variability in cytokeratin expression, or cloning efficiency in the soft agar assay.

The first four patient samples were treated with 10 μM econazole for 24 h. We observed 106- and 510-fold clonogenic depletion of breast cancer colony

formation in two of the samples, while the CFU-GM was reduced only 1.6–6.9-fold. In one sample, breast cancer colony formation was reduced below the limits of detection of the assay.

We evaluated the efficacy of 2-h treatment with econazole plus EGF in four additional samples. We also used three of these samples to evaluate the effect

Figure 8. Detection of contaminating breast cancer cells in PBSC samples from patients with metastatic breast cancer. (A)–(D): Immunofluorescence stain of breast cancer cells using anti-cytokeratin antibody AE1/AE3. (A): MCF-7 positive control. (B)–(D): Positive stain from patient samples. (E): Breast cancer colony cultured from PBSC.

of doxorubicin on both hemopoietic and breast cancer colony formation. As summarized in Table 2, three of the four samples had breast cancer colony-forming cells. In one case, econazole alone reduced breast cancer clonogenicity by 59-fold. The addition of EGF decreased colony formation beyond the limits of detection of the assay (>118-fold). For the other positive samples, in both cases, colony formation was

reduced beyond detectable limits (>30-fold). Effects of the treatment on CFU-GMs varied from 1.2 to 2.9-fold reductions. In no case did the addition of EGF enhance CFU-GM toxicity. Table 3 summarizes the effect of doxorubicin on breast cancer colony formation and CFU-GM content. Doxorubicin was effective in reducing breast cancer colony formation by 30-fold, however, it proved to be quite toxic to CFU-GMs with 18–130-fold decreases in content. In one sample, no CFU-GMs could be detected following Dox treatment.

Discussion

In this study, we have evaluated the effect of the store-operated Ca^{2+} channel blockers econazole and ketotifen on breast cancer cells. We found that both compounds could induce cell death in a sharp concentration-dependent fashion. We demonstrated that co-stimulation of the cells with the agonists EGF or bombesin could enhance cell death by as much as 1 order of magnitude compared to the blockers alone. We also showed that multi-drug resistant MCF-7_{adr} cells displayed equivalent or greater sensitivity to econazole plus EGF than parental MCF-7 cells despite their extraordinary resistance to doxorubicin. We evaluated the effect of econazole or econazole plus EGF on breast cancer cells present in mobilized hemopoietic progenitor cell preparations obtained from patients undergoing high dose chemotherapy with stem cell support. We observed that econazole plus EGF was effective in reducing breast cancer cell clonogenicity by 60–500-fold while hemopoietic progenitor cells were reduced 1.2–6.9-fold. These results, therefore, suggest that AECD and, in particular, the combination of econazole plus EGF, may be useful for purging breast cancer cells from hemopoietic progenitor cell preparations.

Both ketotifen and econazole exhibited relatively sharp dose responses for breast cancer and normal human hemopoietic cells. However, it was possible to find an optimal concentration whereby preferential cell death in breast cancer cells was observed compared to hemopoietic progenitor cells. The optimal treatment appears to be 15 μM econazole plus EGF in serum-free medium for 2 h. Two significant advantages of using the 2-h timepoint are that much lower toxicity is observed in BFU-E cells and the cells can be incubated in serum free conditions. This is important because econazole binds serum proteins very strongly limiting its free concentration in the presence of serum

Table 1. Effect of econazole on colony formation (CFU-GM v.s. breast cancer CFU, 24 h)

Frequency AE1/AE3 ⁺		CFU-GM			CFU breast cancer		
		Ctl.	Econazole*		Ctl.	Econazole*	
Patient	/10 ⁶ MNC	/10 ⁵ MNC	/10 ⁵ MNC	Ratio	/10 ⁶ MNC	/10 ⁷ MNC	Ratio
1	12	7.5	3.9	2.0 ×	15.8	0.31	510 ×
2	0.25	22.1	13.7	1.6 ×	16.9	1.6	106 ×
3	—	12.1	1.75	6.9 ×	—	—	—
4	2	10.3	4.2	2.5 ×	17.7	<1.0	>177 ×

*Concentration of econazole = 10 µM.

Table 2. Effect of econazole on colony formation (CFU-GM v.s. Breast Cancer CFU, 2 h)

Frequency AE1/AE3 ⁺		CFU-GM			CFU breast cancer		
		Ctl.	Econazole*		Ctl.	Econazole*	
Patient	/10 ⁶ MNC	/10 ⁵ MNC	/10 ⁵ MNC	Ratio	/10 ⁶ MNC	/10 ⁷ MNC	Ratio
5	—	30.5	11.7	2.6 ×	—	—	—
+EGF**	—	30.5	25.5	1.2 ×	—	—	—
6	5	129.7	95.7	1.4 ×	11.8	2.0	59 ×
+EGF**	5	129.7	107.3	1.2 ×	11.8	<1.0	>118 ×
7	27.5	70	24.5	2.9 ×	3	<1.0	>30 ×
+EGF**	27.5	70	23.8	2.9 ×	3	<1.0	>30 ×
8	N.D.	42.3	18.5	2.3 ×	3.2	<1.0	>32 ×
+EGF**	N.D.	42.3	19.2	2.2 ×	3.2	<1.0	>32 ×

*Concentration of econazole = 15 µM, serum free.

**EGF (100 ng/ml) was combined with econazole for treatment.

N.D. = not done.

[36]. Strong serum binding probably represents the greatest difficulty for using econazole systemically *in vivo*, however, it is not a limitation *in vitro*.

For both MCF-7 and MDA-MB-231 cells, enhanced cell death was observed when the cells were co-stimulated with EGF or bombesin. For the primary patient samples, activation enhancement of cell death was observed for at least one of the samples. Treatment of two other samples resulted in colony reductions below the limit of detection of the assay. Although these results are preliminary and require further confirmation, they indicate that activation enhancement may be of general use in potentiating the purging effect of econazole.

Although both MCF-7 and MDA-MB-231 cells underwent characteristic morphological changes including nuclear condensation and pyknosis, classical indicators of apoptosis such as DNA laddering and membrane blebbing were not observed. We have also failed to observe caspase activation in these cells in response to AECD (J. Soboloff, Y. Zhang and S.A. Berger unpublished). These results suggest that the

breast cancer cells are not dying by classical apoptosis. The mechanism by which cells undergoing AECD die remains undetermined, however, mitochondrial involvement, while important for a number of cell death pathways appears to be less important for AECD (J. Soboloff and S.A. Berger in preparation). This may in part explain why apoptosis *per se*, while sometimes observed in cells undergoing AECD, is not a universal requirement.

The observation that the multi-drug resistant MCF-7_{adr} cells were not resistant to econazole is very significant. Since drug resistance represents the greatest barrier to effective treatment of breast cancer, it suggests that AECD in general, and the combination of econazole plus EGF in particular, may be of great value in overcoming multi-drug resistance. Previously, we observed that overexpression of bcl-2 also failed to protect cells from econazole plus SLF-induced death [21]. It is, therefore, possible that other common mechanisms of therapeutic resistance may be circumvented by agents that trigger AECD. These possibilities are currently under investigation.

Table 3. Effect of doxorubicin on colony formation (CFU-GM v.s. breast cancer CFU, 2 h)

Patient	Frequency AE1/AE3 ⁺ /10 ⁶ MNC	CFU-GM			CFU breast cancer		
		Ctl.	DOX*	Ratio	Ctl.	DOX*	Ratio
		/10 ⁵ MNC	/10 ⁵ MNC		/10 ⁶ MNC	/10 ⁷ MNC	
5	—	30.5	<1	>30 ×	—	—	—
6	5	129.7	1	130 ×	11.8	4	29.5 ×
7	27.5	70	4	18 ×	3	1	30 ×

*DOX: Doxorubicin (2 µM).

The importance of tumor cell contamination in autologous stem cell products used to support high dose chemotherapy is unknown. AECD appears to be as, or more, effective than the use of monoclonal antibodies plus complement or CD34 selection, while at the same time preserving progenitor cell viability. The feasibility of combining this approach with positive-selection methods and formal comparisons to unpurged, unselected stem cell transplantation awaits the final results of promising ongoing trials of high-dose chemotherapy in the adjuvant treatment of breast cancer.

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References

- Peters WP: High Dose Chemotherapy with Autologous Bone Marrow Transplantation for the Treatment of Breast Cancer. Philadelphia, Lippincott, 1995
- Peters WPJ, Vredenburgh RB, Shpall J, Hussein EJ, Elkordy A, Rubin M, Ross P, Berry M, D: A large, prospective, randomized trial of high-dose combination alkylating agents (CPB) with autologous cellular support (ABMS) as consolidation for patients with metastatic breast cancer achieving complete remission after intensive doxorubicin-based induction therapy (AFM). Proceedings of ASCO, 121, 1996
- Lotz JP, Cure H, Janvier M, Morvan F, Legros M, Asselain B, Guillemot M, Roche H, Gisselbrecht C: Intensive chemotherapy and autograft of hematopoietic stem cells in the treatment of metastatic cancer: results of the national protocol Pegase 04. Hematol Cell Ther 41: 71–74, 1999
- Stadtmauer EA, O'Neill A, Goldstein LJ, Crilley PA, Mangan KF, Ingle JN, Brodsky I, Martino S, Lazarus HM, Erban JK, Sickles C, Glick JH: Conventional-dose chemotherapy compared with high-dose chemotherapy plus autologous hematopoietic stem-cell transplantation for metastatic breast cancer. Philadelphia Bone Marrow Transplant Group. N Engl J Med 342: 1069–1076, 2000
- Peters WP, Dansey RD, Klein JL, Baynes RD: High-dose chemotherapy and peripheral blood progenitor cell transplantation in the treatment of breast cancer. Oncologist 5: 1–13, 2000
- Rodenhuis S: The status of high-dose chemotherapy in breast cancer. Oncologist 5: 369–375, 2000
- Ross AA, Cooper BW, Lazarus HM, Mackay W, Moss TJ, Ciobanu N, Tallman MS, Kennedy MJ, Davidson NE, Sweet D, Winter C, Akard L, Jansen J, Copelan E, Meagher RC, Herzig RH, Klumpp TR, Kahn DG, Warner NE: Detection and viability of tumor cells in peripheral blood stem cell collections from breast cancer patients using immunocytochemical and clonogenic assay techniques. Blood 82: 2605–2610, 1993
- Datta YH, Adams PT, Drobyski WR, Ethier SP, Terry VH, Roth MS: Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. J Clin Oncol 12: 475–482, 1994
- Brugger W, Bross KJ, Glatt M, Weber F, Mertelsmann R, Kanz L: Mobilization of tumor cells and hematopoietic progenitor cells into peripheral blood of patients with solid tumors. Blood 83: 636–640, 1994
- Spyridonidis A, Bernhardt W, Fetscher S, Behringer D, Mertelsmann R, Henschler R: Minimal residual disease in autologous hematopoietic harvests from breast cancer patients. Ann Oncol 9: 821–826, 1998
- Brenner MK, Rill DR, Moen RC, Krance RA, Mirro J, Jr, Anderson WF, Ihle JN: Gene-marking to trace origin of relapse after autologous bone-marrow transplantation. Lancet 341: 85–86, 1993
- Rill DR, Santana VM, Roberts WM, Nilson T, Bowman LC, Krance RA, Heslop HE, Moen RC, Ihle JN, Brenner MK: Direct demonstration that autologous bone marrow transplantation for solid tumors can return a multiplicity of tumorigenic cells. Blood 84: 380–383, 1994
- Freedman A, Friedberg JW, Gribben J: High-dose therapy for follicular lymphoma. Oncology (Huntingt) 14: 321–326, 329; discussion 330–332, 338, 2000
- Shpall EJ, Jones RB, Bast RC, Jr, Rosner GL, Vandermark R, Ross M, Affronti ML, Johnston C, Eggleston S, Tepperburg M, Coniglio D, Peters WP: 4-Hydroperoxycyclophosphamide purging of breast cancer from the mononuclear cell fraction of bone marrow in patients receiving high-dose chemotherapy and autologous marrow support: a phase I trial. J Clin Oncol 9: 85–93, 1991

15. Anderson IC, Shpall EJ, Leslie DS, Nustad K, Ugelstad J, Peters WP, Bast RC, Jr: Elimination of malignant clonogenic breast cancer cells from human bone marrow. *Cancer Res* 49: 4659–4664, 1989
16. Passos-Coelho J, Ross AA, Davis JM, Huelskamp AM, Clarke B, Noga SJ, Davidson NE, Kennedy MJ: Bone marrow micrometastases in chemotherapy-responsive advanced breast cancer: effect of *ex vivo* purging with 4-hydroperoxycyclophosphamide. *Cancer Res* 54: 2366–2371, 1994
17. Fauth F, Martin H, Sonnhoff S, Bialleck H, Wiesneth M, Mihanovic B, Hoelzer D: Purging of G-CSF-mobilized peripheral autografts in acute leukemia with mafosfamide and amifostine to protect normal progenitor cells. *Bone Marrow Transplant* 25: 831–836, 2000
18. Shpall EJ, Jones RB, Bearman SI, Franklin WA, Archer PG, Curiel T, Bitter M, Claman HN, Stemmer SM, Purdy M, Myers SE, Hami L, Taffs S, Heimfeld S, Hallagan J, Berenson RJ: Transplantation of enriched CD34-positive autologous marrow into breast cancer patients following high-dose chemotherapy: influence of CD34-positive peripheral-blood progenitors and growth factors on engraftment. *J Clin Oncol* 12: 28–36, 1994
19. Chabannon C, Cornetta K, Lotz JP, Rosenfeld C, Shlomchik M, Yanovitch S, Marolleau JP, Sledge G, Novakovitch G, Srour EF, Burtress B, Camerlo J, Gravis G, Lee-Fischer J, Faucher C, Chabbert I, Krause D, Maraninchi D, Mills B, Kunkel L, Oldham F, Blaise D, Viens P: High-dose chemotherapy followed by reinfusion of selected CD34+ peripheral blood cells in patients with poor-prognosis breast cancer: a randomized multicentre study. *Br J Cancer* 78: 913–921, 1998
20. Kennedy MJ: Peripheral blood progenitor cell mobilization: a clinical review. *Pharmacotherapy* 18: 3S–8S, 1998
21. Gommerman JL, Berger SA: Protection from apoptosis by steel factor but not interleukin-3 is reversed through blockade of calcium influx. *Blood* 91: 1891–1900, 1998
22. Fox SB, Smith K, Hollyer J, Greenall M, Hastrich D, Harris AL: The epidermal growth factor receptor as a prognostic marker: results of 370 patients and review of 3009 patients. *Breast Cancer Res Treat* 29: 41–49, 1994
23. Hynes NE: Amplification and overexpression of the erbB-2 gene in human tumors: its involvement in tumor development, significance as a prognostic factor, and potential as a target for cancer therapy. *Semin Cancer Biol* 4: 19–26, 1993
24. Peyrat JP, Bonnetterre J, Hondermarck H, Hecquet B, Adenis A, Louchez MM, Lefebvre J, Boilly B, Demaille A: Basic fibroblast growth factor (bFGF): mitogenic activity and binding sites in human breast cancer. *J Steroid Biochem Mol Biol* 43: 87–94, 1992
25. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244: 707–712, 1989
26. el Yazidi I, Boilly-Marier Y: Production of acidic and basic fibroblast growth factor by the hormone-independent breast cancer cell line MDA-MB-231. *Anticancer Res* 15: 783–790, 1995
27. Mossman T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Immunol Meth* 65: 55, 1983
28. Bian X, Hughes FM, Jr, Huang Y, Cidlowski JA, Putney JW, Jr: Roles of cytoplasmic Ca^{2+} and intracellular Ca^{2+} stores in induction and suppression of apoptosis in S49 cells. *Am J Physiol* 272: C1241–1249, 1997
29. Putney JW, Jr, McKay RR: Capacitative calcium entry channels. *Bioessays* 21: 38–46, 1999
30. Kueng WM, Silber E, Novak I, Eppenberger U: Effects of hormones and growth factors on the growth of six human breast cancer cell lines in defined media. *Contribut Oncol* 23: 26–32, 1986
31. Dong XF, Berthois Y, Martin PM: Effect of epidermal growth factor on the proliferation of human epithelial cancer cell lines: correlation with the level of occupied EGF receptor. *Anticancer Res* 11: 737–743, 1991
32. Bold RJ, Ishizuka J, Yao CZ, Townsend CM, Jr, Thompson JC: Bombesin stimulates *in vitro* growth of human breast cancer independent of estrogen receptors status. *Anticancer Res* 18: 4051–4056, 1998
33. Yano T, Pinski J, Groot K, Schally AV: Stimulation by bombesin and inhibition by bombesin/gastrin-releasing peptide antagonist RC-3095 of growth of human breast cancer cell lines. *Cancer Res* 52: 4545–4547, 1992
34. Gottesman MM, Pastan I: Biochemistry of multi-drug resistance mediated by the multi-drug transporter. *Annu Rev Biochem* 62: 385–427, 1993
35. Lehnert M: Clinical multi-drug resistance in cancer: a multifactorial problem. *Eur J Cancer* 32A: 912–920, 1996
36. Gamberucci A, Fulceri R, Benedetti A, Bygrave FL: On the mechanism of action of econazole, the capacitative calcium inflow blocker. *Biochem Biophys Res Commun* 248: 75–77, 1998

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