Purpose: Overexpression of survivin in breast cancer cells is associated with aberrant inhibition of apoptosis which leads to massive proliferation of cancer cells. Downregulation of survivin by the anticancer agent prodigiosin can efficiently induce apoptosis in cancer cells.

Methods: The levels of survivin expression in breast cancer stem like side population (SP) cells were assessed. Analyzed were also the rate of apoptosis, drug resistance and the efficiency of clone formation of breast cancer SP cells after treatment with prodigiosin.

Results: Breast cancer samples contained about 2.7% of cancer stem like SP cells which possessed elevated mRNA expression of stem cell proteins Oct-4, EpCAM and ABC transporter ABCG2, essential for the maintenance of SP cells. Furthermore, the SP cells displayed overexpression of survivin in conjunction with reduced apoptosis and increased multidrug resistance. After treatment with prodigiosin, the SP cells became more sensitive to apoptosis and to several chemotherapeutic agents.

Conclusion: These data suggest that increased expression of survivin in SP cells is one of the major factors involved in apoptosis and resistance to chemotherapy.

Key words: apoptosis, drug resistance, prodigiosin, side population cells, survivin

Introduction

Breast cancer is the most common malignancy in females and the second leading cause of cancer mortality worldwide [1]. The average survival rate of breast cancer patients after diagnosis at metastatic stage is 5 years [2]. Significant risk factors of breast cancer include early menarche, late menopause, late age at first pregnancy, obesity, postmenopausal obesity, oral contraceptives, hormone replacement therapy and family history [3-5]. The biological profile of breast cancer includes screening of hormone receptors such as oestrogen receptor (ER), progesterone receptor (PR) and HER-2 overexpression. Despite recent advances in breast cancer therapies, therapy failures are frequent and complete tumor eradication is still a target to be achieved. Breast cancer is often treated with combination of radiotherapy, hormone therapy and chemotherapy. Accumulating evidence suggests that breast cancer treatment failure is mainly caused by the presence of a rare and small population of cancer stem cells (CSCs). These CSCs evade the treatment regimen and are responsible for minimal residual disease (MRD) [6]. CSCs possess the characteristics associated with stem cells such as self-renewal, exhibit high in vivo tumorigenicity, differentiation potential, multidrug and apoptosis resistance [7]. Cells that exclude Hoechst 33342 dye are referred to as SP...
cells. These cells share the characteristics of CSCs, they are specifically enriched for tumor initiation, express stem-like genes and resistance to chemotherapeutic drugs due to overexpression of ATPase binding cassette (ABC) transporters such as ABCB1 (MDR1), ABCC1, ABCG2 (BCRP1) [8].

The most important characteristic feature of cancer cells is downregulation of programmed cell death (apoptosis) which plays a crucial role in tumorigenesis [9,10]. A recent study by Ambrosini et al. has reported that increased expression of survivin (unique inhibitor of apoptosis/IAP) contributed to apoptosis resistance of cancer cells and hence it acts as an anti-apoptotic factor like bcl-2 [11]. The IAP proteins are highly evolutionarily conserved and they efficiently block the conserved steps in apoptosis pathways such as inhibition of caspases [11,12]. Previous studies in breast cancer cell lines reported that enhanced expression of survivin can negatively impact tumor cell apoptosis and overall survival [13,14]. Furthermore, downregulation of survivin results in enhanced apoptosis and chemosensitization of cancer cells treated with paclitaxel [14,15]. Prodigiosin (2-methyl-3-pentyl-6-methoxyprodiginine) belongs to the family of tripyrrole red pigments and possesses antimetastatic and proapoptotic properties [16,17]. The proapoptotic effect of prodigiosin is well demonstrated in several cell lines including breast cancer where it was shown that survivin is efficiently targeted by prodigiosin treatment [14].

It is well known that CSCs are highly multidrug-and apoptosis-resistant in different cancers. Therefore, in the current study we assessed the level of survivin expression in breast cancer stem like SP cells. We also analyzed the rate of apoptosis, drug resistance and clone formation efficiency of breast cancer SP cells after treatment with prodigiosin and the results of this study are described in detail below.

Methods

Cancer samples and cell culture

Invasive breast carcinoma samples were collected at the time of surgery of patients who underwent potentially curative resection and did not receive any form of treatment prior to surgery. All patients were female and their mean age was 42.1 years (range 35-46). Their tumor stage (T stage) was II/III with lymph node metastasis (N+). The collected tumor samples were mechanically dissociated and enzymatically digested in a 1:1 solution of Type III collagenase/hyaluronidase for 30 min at 37°C and incubated at 37°C for 2 hrs in a shaking bath. In order to remove the clumps, sterile gauzes (pore diameter sizes: 200 mesh) were used and erythrolysis was performed in hypotonic solution (0.2% NaCl) and subsequently by 1.2% NaCl to stop lysis. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS), supplemented with antibiotics and maintained in T-75 flasks at 37°C in a humidified 5% CO2 and 95% air atmosphere. On becoming 90% confluent, cells were removed from the culture flask using Trypsin-EDTA (0.25% - 53 mM EDTA), washed, suspended in 10% DMEM and centrifuged at 5000 rpm for 6 min. Then, they were resuspended in 10% DMEM and counted using hemocytometer.

Labeling with Hoechst 33342

Study group: Control: cells+Hoechst 33342 (N=5); Drug treated: cells+Verapamil+Hoechst 33342 (N=5).

Cells in staining medium (approx 106 cells/ml in 10% DMEM) were labeled with Hoechst 33342 stock (sigma)-bis-benzimide (5μl/ml) either with dye alone or in combination with drug (verapamil - 0.8μl/ml). The cells were mixed and placed in water bath at 37°C for 90 min. After 90 min, cells were centrifuged (2000 rpm for 10 min at 4°C) and resuspended in 500μl of Hanks balanced salt solution (HBSS, Sigma, St.Louis, MO, USA) containing 10mM HEPES (Sigma, St.Louis, MO, USA). Finally, the cells were counterstained with PI (propidium iodide) 2μg/ml per sample at 4°C for 5 min. Cells were then filtered through a 50μm nylon mesh (BD) to remove cell clumps into labeled FACS tubes. Separate tubes with medium (10% DMEM) were labeled with Hoechst 33342 stock (Sigma) containing 10mM HEPES (Sigma, St.Louis, MO, USA). The Hoechst 33342 dye was excited at 355 nm and its dual-wavelength fluorescence was analyzed (blue, 450 nm; red, 675 nm).

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from FACS-sorted SP and non-SP using the Ambion RNAqueous®-MicroK-it (Applied Biosystems, Warrington, UK). cDNA was synthesized using the Bioline cDNA synthesis kit (Bioline, London, UK). RT-PCR was performed using 2-5 μl cDNA and 2X TaqMan Gene Expression Mastermix (Applied Biosystems, Warrington, UK) in 30 μl reaction volumes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the standard endogenous expression. The primers were designed to encompass an exon junction to prevent templating from possibly contaminated genomic DNA. Primer sequences were: for Survivin: F; TCC ACT GCC CCA CTG AGA AC and R; TGG TTC CCA GCC TTC CA [14]; ABCG2: F, AGC TGC AAG GAA AGA TCC AA and R, TCC AGA CAC ACC ACG GAT AA; for Oct-4: F, ATC CTG GGG GTT CTA TTT GG and R, CTC CAG GTT GCC TCT CAC TC; for EpCAM: F, CTG CCA AAT GTT TGG TGA TG and R, ACG GTT TGT GAT CTC CTT CT; and for GAPDH: F, ATG TCG TGG AGT CTA CTG...
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GC and R, TGA CCT TGC CCA CAG CCT TG. PCR conditions: 52°C for 2 min and 95°C for 6 min followed by 35 cycles of 15 sec at 95°C, 30 sec at 58°C, and 45 sec at 72°C. GAPDH was used as housekeeping gene. The amplified products were separated by electrophoresis on ethidium bromide-stained 1.2% agarose gels. Band intensity was measured by Image J from three independent experiments. The values presented in the graph are the average values of three independent experiments.

Chemoresistance assay

Approximately 1x10^3 cells/plate were cultured in 96-well plates and treated with the chemotherapeutic drugs with or without prodigiosin (as previously described concentration ref). The concentration of the drugs used was: 5-fluorouracil (5-FU) 10 μg/ml, paclitaxel 30 ng/ml and cisplatin 5 mg/ml. The mean value of OD_{450} obtained was presented as a graph. Cell resistance in both groups was calculated using the following formula: Cell resistance rate (%) = (experimental group OD_{450} value/control group OD_{450} value) x100. The values presented in the graph are the average of three independent experiments.

Western blot analysis

For western blot analysis, proteins were extracted from the SP and non-SP cells, and the protein concentration was determined using the Bradford assay [18]. Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transfer to a membrane, the gels were treated with the primary antibodies ABCG2, Bcl-2, GAPDH and survivin (rabbit anti-human) and the secondary antibody (goat anti-rabbit IgG with alkaline phosphatase markers) and a chemiluminescence reagent. Blots were detected and scanned by using a densitometer (Biorad GS-710, Hercules, CA, USA). GAPDH was used as a loading control.

Clone formation efficiency

The sorted SP and non-SP cells were placed at a density of 1000 cells/ml resuspended in tumor sphere medium consisting of serum-free 1:1 mixture of Ham's F-12/DMEM, N2 supplement, 10 ng/ml human recombinant bFGF, and 10 ng/ml EGF and subsequently cul-

Figure 1. Analysis of side population (SP) cells in breast cancer samples. A: Cells stained using Hoechst 33342 dye and analyzed by flow cytometry. SP cells of 2.7% are outlined (gated population) from main population cells. B: The percentage of SP cells is significantly reduced (0.7%) in the presence of verapamil. C: Expression of ABCG2 and stem cell markers in SP and non-SP cells by RT-PCR. Quantification graph showing the elevated mRNA expression of ABCG2, Oct-4, and EpCAM genes in SP cells. GAPDH is used as a housekeeping gene. The bar represents standard deviation. **p < 0.01.
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Cultured in ultra low attachment plates for about 2 weeks. SP and non-SP cells sorted were seeded at a low density of 20 cells/L and the number of generated spheres (>100 ml) was counted after 7 days of culture. The values presented in the graph are the average values of three independent experiments.

TUNEL assay

The rate of apoptosis was analyzed by TUNEL assay by using detection kit (Boehringer Mannheim, Germany). 2x200 cells were randomly counted from three independent experiments.

Results

We identified about 2.7% SP cells from breast cancer samples by FACs-based Hoechst 33342 dye exclusion technique (Figure 1A). Upon treatment with verapamil (ABC transporter inhibitor) the SP cells were significantly reduced to 0.7% (Figure 1B), which confirms that the dye exclusion property of SP cells actively involves ABC transporters. In addition, RT-PCR analysis revealed that SP cells had relatively enhanced mRNA expression of stem cell surface genes such as EpCAM and Oct-4 (Figure 1C) which are essential for the self-renewal of SP cells.

Interestingly, Western blot showed that the expression of antiapoptotic factors such as survivin, bcl-2 and ABC transporter protein ABCG2 were relatively higher in SP cells which are responsible for apoptosis and chemoresistance (Figure 2A). The prodigiosin-treated SP cells had significantly reduced expression of survivin, bcl-2 and ABCG2 as well (Figure 2B). Addition of prodigiosin repressed the survivin at the transcription level and this was confirmed by RT-PCR analysis where the mRNA level of survivin was significantly reduced (Figure 2C).

Next, we investigated the chemoresistance efficiency of SP cells to drugs such as 5-flurouracil, paclitaxel and cisplatin. The SP cells showed in-

Figure 2. Western blot analysis of protein expression levels in side population (SP) and non-SP cells. A: SP cells showing increased expression of ABCG2, survivin and bcl-2. B: Upon treatment with prodigiosin the expression of ABCG2, survivin and bcl-2 are significantly reduced in SP cells. Equal concentrations of protein were loaded per lane. GAPDH is used as a loading control. C: RT-PCR analysis showing that mRNA expression of survivin is significantly reduced in SP cells upon addition of prodigiosin. SP+ and non-SP+ are the prodigiosin-treated cells. The bar represents standard deviation. *p<0.05; ** p<0.01.
increased resistance to these drugs and they showed enhanced survival compared to non-SP cells (Figure 3A). However, after treatment with prodigiosin, SP cells became significantly sensitive to the chemotherapeutic drugs and their overall survival rate was reduced near to non-SP cells (Figure 3B). Similarly, the apoptosis rate of SP cells was significantly enhanced after treatment with prodigiosin (compare Figure 4A and 4B).

Finally we examined the clone formation efficiency of SP cells. As expected, the SP cells could generate tumor spheres more rapidly than non-SP cells (Figure 5A). After prodigiosin treatment, the clone formation efficiency of SP cells was greatly reduced (Figure 5B). Therefore, our data clearly indicate that breast cancer SP cells were responsible for drug resistance, therapy failure and tumorigenesis. Furthermore, the enhanced expression of survivin played a crucial role in chemotherapy- and apoptosis-resistance of SP cells, as its downregulation by prodigiosin treatment turned SP cells more sensitive to apoptosis and chemotherapeutic drugs.

**Discussion**

In the current study we demonstrated that upregulation of survivin in breast cancer SP cells contributed to apoptosis resistance and multidrug resistance. Furthermore, the effect of survivin was compromised by prodigiosin and therefore SP cells became more sensitive to apoptosis and chemotherapeutic drugs.

**Figure 3.** Comparison of cell survival rate of side population (SP) cells and non-SP cells. **A:** SP cells showed strong resistance to 5 fluorouracil (5-FU), cisplatin and paclitaxel and they had increased survival compared to non-SP cells. **B:** With further treatment with prodigiosin, the survival rate of SP cells was significantly reduced and was almost the same as the non-SP cells. The x-axis represents time, while the y-axis indicates the corresponding optical density (OD) value at 450 nm. The bar represents standard deviation. *p<0.05; **p<0.01.

**Figure 4.** Analysis of cell death rate in side population (SP) and non-SP cells. **A:** Reduced apoptosis was observed in SP cells. **B:** After treatment with prodigiosin, the number of apoptosis-positive SP cells increased significantly. 2x200 cells were counted randomly. The bar represents standard deviation. *p<0.05; **p<0.01.
gies are able to target the cancer cells efficiently, leaving however the CSCs unaffected and resulting thus in cancer therapy failure and MRD. CSCs are characterized by high self-renewal capacity, multidrug and apoptosis resistance and high ability of differentiation. Plethora of studies have reported that SP cells are enriched CSCs as they share all the remarkable features of CSCs. In addition, the identification and characterization of SP cells helps to target and kill the CSCs so that complete eradication of a refractory tumor could be achieved. In the present work we identified about 2.7% of SP cells in breast cancer samples which possess higher mRNA expression level of ABCG2 (ABC transporter), and stem cell surface genes (Oct-4 and EpCAM) compared with non-SP cells. Several studies on solid tumors have shown that the presence of SP cells and upregulation of ABC transporters and stem cell surface proteins play a major role in drug resistance and massive proliferation of cancer cells [19,20]. The OCT-4 gene belongs to the family of POU transcription factors involved in cell proliferation and maintenance of CSCs by activating the Oct-4/Tcf1/Akt1 pathway [21,22]. Similarly, overexpression of epithelial adhesion molecule (EpCAM) showed high tumorigenic activity [23]. In line with these findings, our data suggest that high expression level of ABCG2, EpCAM and OCT-4 in SP cells might act as a crucial factor for drug resistance, tumorigenesis and metastasis.

The most remarkable feature of cancer cells are their ability to resist apoptosis which allows cancer cells to undergo rapid proliferation and enhanced survival. In recent years, it was shown that survivin is a potential inhibitor of apoptosis and its overexpression prevents cancer cells from programmed cell death [13,14]. Prodigiosin has been considered as an anticancer agent as it efficiently induces apoptosis of cancer cells [16,17]. It has been demonstrated that survivin expression is efficiently downregulated by treatment with prodigiosin [14]. Similar to these findings, we also showed that survivin expression is enhanced in SP cells than in non-SP cells and obviously these SP cells are highly resistant to apoptosis and chemotherapeutic drugs. Therefore, survivin acts as a potential antiapoptotic factor like bcl-2. In addition, treatment with prodigiosin induces apoptosis of SP cells concomitantly with increased sensitization to chemotherapeutic drugs. It was previously demonstrated that prodigiosin efficiently targets the survivin, activates the caspases’ apoptotic pathways and induces higher sensitization to paclitaxel [14]. However, the precise molecular mechanism and downstream signaling pathways involved in survivin-mediated resistance to apoptosis of cancer cells still needs to be elucidated. The regulation of tumorigenesis mediated by survivin involves both transcriptional and post-translational levels and prodigiosin was suggested to repress survivin at the transcriptional level [24]. The present study also showed that the massive proliferation of SP cells was reduced after treatment with prodigiosin. Taken together, these data suggest that prodigiosin can be a potential anticancer agent, however how prodigiosin-mediated survivin repression and chemosensitization of cancer cells remains speculative.

In summary, our data clearly demonstrated that increased expression of survivin in SP cells is significant for drug resistance and survival of breast cancer cells.
cells is one of the major factors involved in apoptosis and chemotherapy resistance. Further studies concerning the molecular mechanisms of survivin are necessary for they could lead to the development of novel targeted anticancer agents which could effectively target and downregulate survivin, inducing and contributing thus to CSCs death and prevention of tumor relapse.

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