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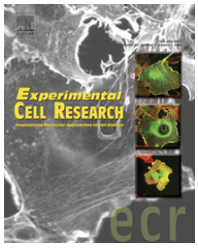

Regular Article

Endonuclease G promotes cell death of non-invasive human breast cancer cells

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ABSTRACT

The invasiveness of breast cancer cells was shown to be associated with the suppressed ability to develop apoptosis. The role of cell death DNases/endonucleases has not been previously examined in relation with the invasiveness of breast cancer cells. We have compared the activity of the endonucleases in seven human breast cancer cell lines different in the level of invasiveness and differentiation. The invasiveness of cell lines was confirmed by an *in vitro* Matrigel-based assay. The total endonuclease activity in the differentiated non-invasive (WDNI) cell lines was higher than that in the poorly differentiated invasive (PDI) cells. The expression of EndoG strongly correlated with the degree of estrogen receptor expression and showed an inverse correlation with vimentin and matrix metalloproteinase-13. The EndoG-positive WDNI cells were more sensitive to etoposide- or camptothecin-induced cell death than EndoG-negative PDI cells. Silencing of EndoG caused inhibited of SK-BR-3 WDNI cell death induced by etoposide. Human ductal carcinomas *in situ* expressed high levels of EndoG, while invasive medullar and ductal carcinomas had significantly decreased expression of EndoG. This correlated with decreased apoptosis as measured by TUNEL assay. Our findings suggest that the presence of EndoG in non-invasive breast cancer cells determines their sensitivity to apoptosis, which may be taken into consideration for developing the chemotherapeutic strategy for cancer treatment.

Introduction

Increased breast cancer cell invasiveness is associated with progression and a poor prognosis in patients [1,2]. Commonly, invasive carcinomas have less apoptosis and are more resistant to chemotherapy [3–5]. Several studies showed decreased apoptosis in breast cancer cells in association with cancer progression, invasiveness and metastases [3,4]. Histological analysis of human breast carcinomas showed that poorly differentiated breast tumors, but not well-differentiated

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breast tumors, have decreased apoptosis, which seems to be important in carcinogenesis and progression [5].

Apoptosis is known to play a major role in carcinogenesis and tumor development. Suppression of apoptosis was proposed to cause inappropriate survival of genetically aberrant cells during carcinogenesis [6]. The neoplastic transformation of mammary epithelial cells has been related to decreased apoptotic cell death [7].

DNA fragmentation, a commonly accepted marker and metabolic pathway of apoptotic cell death, is generated by a member of the recently identified group of cell death endonucleases [8]. Anticancer drugs induce apoptosis in cancer cells through endonuclease-mediated DNA fragmentation [9,10], while the inhibition of endonucleases has a protective effect on cancer cell death [10]. Cell death endonucleases include deoxyribonuclease I (DNase I) [11], deoxyribonuclease II (DNase II) [12], endonuclease G (EndoG) [13], caspase-activated DNase (CAD) [14] and DNase gamma [15]. All these enzymes, except DNase II, require Ca^{2+}, Mg^{2+} or Mn^{2+} ions for their activity. The enzymes differ in certain catalytic characteristics and DNA sequence specificity; however, they produce a similar type of DNA damage, which consist of single- and double-strand DNA breaks [8,16]. While being considered as downstream effectors of apoptotic cascades, the cell death endonucleases can cause DNA fragmentation and irreversible cell death seemingly by acting alone after activation, overexpression or introduction into a living cell [11–15].

The role of cell death endonucleases in any cancer, including breast cancer, has not been investigated yet. The present study was aimed to determine the changes of endonucleases in breast cancer cells that are different by their degrees of differentiation and invasiveness. Our study showed that normal breast cells express DNase I and a Mn-dependent endonuclease similar to EndoG. We compared four cell lines that are well-differentiated non-invasive (WDNI) cells (MCF-7, AU-565, ZR-75-1 and SK-BR-3) with three poorly differentiated invasive (PDI) cell lines (HCC1143, HCC1954 and HCC1395). The expression of ER was used as a marker of breast cell differentiation, whereas vimentin, matrix metalloproteinase-13 (MMP-13) and Matrigel-invasion assay were used to measure invasiveness. We found that DNase I activity determined by substrate gel electrophoresis was present only in well-differentiated MCF-7 breast cancer cells and tumor xenografts produced by these cells. Unexpectedly, we found that EndoG determined by using Western blotting was expressed in WDNI cells, whereas expression in PDI cells was extremely low. We further demonstrated that EndoG-positive cells are more sensitive to cell death induced by etoposide. We also showed that human invasive carcinomas have a decreased expression of EndoG and decreased endonuclease-mediated apoptosis. Taken together, our data suggest that the presence of EndoG in non-invasive breast cancer cells determines their sensitivity to apoptosis.

Materials and methods

Animals

DNase I-knockout (KO) mice (CD-1 background) were provided by H. Mannherz of the University of Bochum, and T. Moroy of the University of Essen, Germany. The mice were bred as heterozygotes and genotyped by PCR as suggested by Napirei et al. [17]. Mammary glands were obtained from fourteen days pregnant female mice. Adult female BALB/c-*nu/nu* mice, 8–10 weeks of age, were used as host animals for xenografted tumors. The tumors were initiated from monolayer cell cultures of MCF-7 and ZR-75-1 cells, as well as BT-474 (ATCC # HTB-20) cells. Approximately 3.5×10^5 cells suspended in 10 μl of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution were inoculated intradermally into the left mouse flank. The animals received a weekly percutaneous administration of 100 μg of 17β estradiol (Sigma) in 10 μl of ethanol until tumors reached approximately 150–200 mm^3. All experiments with animals were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System.

Cells and treatment

All breast cancer cell lines were obtained from the American Type Culture Collection (ATCC). Well-differentiated cell lines included MCF-7 (ATCC # HTB-22), AU-565 (ATCC # CRL-2351), ZR- 75-1 (ATCC # CRL-1500) and SK-BR-3 (ATCC # HTB-30). Poorly differentiated cells were: HCC1143 (ATCC # CRL-2321), HCC1954 (ATCC # CRL-2338) and HCC1395 (ATCC # CRL-2324). Well-differentiated cell lines were expressing ER or HER-2/neu, and were initially isolated from non-invasive tumors. Poorly differentiated cell lines did not express ER (except HCC1395 cells), progesterone receptor or HER-2/neu. All cells were maintained in media and growth conditions (5% CO_2–95% air in humified incubator at 37°C) suggested by the supplier. To induce cell death, camptothecin (Sigma-Aldrich, St. Louis, MO) or etoposide (Sigma-Aldrich) was added to serum-free media for 24 h. After exposure to camptothecin or etoposide, the lactate dehydrogenase (LDH) release assay kit (Promega, Madison, WI) was used. Toxicity was expressed as the ratio of LDH release in the medium of treated cells media to that of the maximal LDH release.

EndoG siRNA silencing

SK-BR-3 cells were seeded in 6- or 96-well plates and grown to 60–70% confluence. To knockdown EndoG mRNA, cells were transfected with designed siRNA duplexes (sense siRNA 5′-AUGCCUGGAACAACCUGGAdTdT-3′ antisense siRNA 3′-UCCAGGUUGUUCCAGGCAUdTdT-5′) or Control Non-Targeting siRNA #1 (Dharmacon, Lafayette, CO). The cells were treated with 50 nM siRNA mixed with TransIT-TKO transfection reagent (Mirus, Houston, TX) according to manufacturer's recommendations in serum-free medium for 24 to 96 h. EndoG mRNA expression was measured using real-time RT-PCR of the extracted total RNA. In experiments with etoposide treatments, the cells were transfected with anti-EndoG siRNA in 96-well plates for 72 h. The medium was exchanged to serum-free medium and cells were exposed with etoposide for additional 24 h.

RNA extraction and real-time RT-PCR

The total RNA was extracted using RNeasy Mini kit from Qiagen as suggested by the manufacturer. The quality of RNA