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Linking the ceramide synthases (CerSs) 4 and 5 with apoptosis, endometrial and colon cancers

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ABSTRACT

Ceramide synthases (CerSs) also known as Longevity Assurance (LASS) genes belong to a family of six related genes. CerS gene products have been shown to produce ceramide, hence their name CerSs. Ceramide is a bio-effector molecule, belonging to the family of sphingolipids (SLs), which are important components of cell membranes, and has been implicated in cancer and apoptosis. Cancer still remains the second leading cause of death, both globally and in South Africa. The proper regulation of the balance between cell growth and cell death is essential for cellular homeostasis. Failure to properly regulate this balance may lead to pathologic conditions such as cancer development. CerSs have been implicated in cancer biology, especially in apoptosis, through the action of ceramide. Although knowledge of the role that CerSs play in cancer biology is advancing, the precise roles of distinct CerSs in different cancers are not yet fully understood, especially the roles of CerS4 and CerS5 in endometrial and colon cancers.

The aim of this study was to investigate the link of CerS4 and CerS5 in apoptosis and, thus in cancers of the endometrium and colon, which are amongst the most prevalent cancers globally. Apoptosis was induced using anastrozole in endometrial cells and 5-FU in colon cells. Fluorescence activated cell sorting was used to analyse and quantify apoptosis and total RNA was extracted from both treated and untreated cells. Quantitative relative expression of CerS4 and CerS5 mRNA was then determined in all cells (treated and untreated), normalised to β-actin. Bio-informatics was used to compare CerS4 and CerS5 sequences.

The endometrial cancer cells were more prone to apoptosis compared to their non-cancerous counterparts, while the colon cancer cells were more responsive to apoptosis induction after 48 h, especially the HT-29 cells. Using quantitative real-time PCR, both CerS4 and CerS5 were shown to be up-regulated in endometrial and colon cancer cells. Apoptosis induction resulted in down-regulation of CerS4 and CerS5 in endometrial and colon cancers. These findings implicate these genes in cancer and apoptosis. Whether these genes play pro- or anti-apoptotic roles in cancers of the endometrium and colon is not conclusive at this stage. It may also be possible that these genes could exert opposing roles in the same or different tissues. Targeting this family of genes and understanding their precise individual roles in different types of cancer, are a promising therapeutic tool to new anti-cancer drug discovery or improving existing treatments.

1. Introduction

Cancer is the second leading cause of mortality worldwide, and in SA (Parkin et al., 2008). It is estimated by the WHO that 1 in 8 individuals will develop cancer during their lifetime. Therefore cancer still remains a public health problem, both globally and locally. The development of cancer may be hereditary or spontaneous, with severe genetic changes occurring during cancer development (Parkin et al., 2005). Endometrial cancer and colon cancer, which are amongst the most prevalent cancers, have also been previously linked to genetic changes (Matsumoto et al., 2008; Wu et al., 2004). Both hereditary and spontaneous processes lead to alterations in the genetic cascade. Endometrial cancer is one of the most common cancers seen in South African women (CANSA, 2012). Furthermore, colon cancer, amongst other solid tumours, is one of the most poorly treated (Senkal et al., 2010). Disturbances in a number of biological processes, such as ceramide synthesis and signalling have been implicated in many cancers, including endometrial and colon cancers (Knapp et al., 2010; Ogretmen, 2006; Wu et al., 2004). Ceramide is a bio-effector molecule which forms the backbone of the sphingolipid (SL) family (Hartmann et al., 2012; Ogretmen, 2006). SLs are important components of cell-membranes, and therefore play a critical role in signal transduction, especially ceramide as a second messenger (Ogretmen, 2006; Pewzner-Jung et al., 2006).

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Ceramide can be produced via two distinct pathways. Firstly, by the hydrolysis of sphingomyelin (SM) through sphingomyelinase (SMase), and secondly, ceramide can be produced de novo by a family of genes known as ceramide synthases (CerSs), which consists of six members, CerS1 to CerS6 (Levy and Futerman, 2010; Pewzner-Jung et al., 2006). The link between the two pathways of ceramide production (by SMase and by CerSs) is poorly understood. De novo synthesis of ceramide occurs in the endoplasmic reticulum (Pewzner-Jung et al., 2006). Ceramide is subsequently transported to the Golgi by either vesicular trafficking or the ceramide transfer protein CERT. Once in the Golgi apparatus, ceramide can be further metabolized to other sphingolipids, such as sphingomyelin (Hannun and Obeid, 2008). It is therefore, not yet known if the ceramide molecules produced via these two pathways exert the same biological effects in the same or different tissues.

A controlled balance between cell growth and cell death is essential for cellular homeostasis (Parkin et al., 2005). Ceramide has been shown to play an important role in determining cell fate (Ogretmen, 2006). Furthermore, it has been shown that disturbances in ceramide signalling may lead to alterations in apoptotic signalling, and eventually to cancer development (Senkal et al., 2007). Moreover, the CerS family including CerS4 and CerS5 has been implicated in other cancers, such as breast cancer (Senkal et al., 2007). In addition, it has been previously shown that different members of this family may exert opposing roles in the same cell. This for example was demonstrated by Senkal et al. (2007), when they showed the pro-apoptotic role of CerS1 and the anti-apoptotic role of CerS6 in head and neck squamous cell carcinoma (HNSCC). Additionally, little is known about the alternate isoforms of CerSs, for example, little is known about the pro-apoptotic role of CerS1 isoform 2, as isoform 1 has been recently documented in HNSCC (Meyers-Needham et al., 2011). The precise roles of CerS4 and CerS5 in endometrial and colon cancers are not yet fully understood. Recently, it has emerged that ceramide synthases act as targets for chemotherapeutic drugs. For example, the expression of ceramide synthase 1, which predominantly synthesises C18-ceramide, has been shown to sensitise tumour cells to several chemotherapeutic drugs including cisplatin, gemcitabine, doxorubicin and vincristine (Henry et al., 2013; Senkal et al., 2007). However, little is known about the exact anti-tumour therapeutic roles of CerS4 and CerS5 in endometrium and colon cancers.

This study aimed to investigate the link of CerS4 and CerS5 in response to apoptosis inducing drugs in endometrial and colon cancers using FACS, quantitative RT-PCR, and the bio-informatics analysis of the amino acid sequences of these two proteins.

2. Materials and methods

2.1. Cell culture and apoptosis detection by fluorescence activated cell sorting (FACS)

The endometrial cells (GMMe and Hec-1A) were obtained from the American Type Culture Collection, and the colon cells (Caco-2 and HT-29) were obtained from Highveld Biologicals (Pty Ltd). Cells were cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were cultured at 37 °C in an atmosphere containing 5% CO_2. Endometrial cells were treated with 2 μM anastrozole for 24 h and colon cells were treated with 5 μM 5-FU for 24 h and 48 h. After treatment, cells were trypsinized and washed twice with PBS. Using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences), cells (1×10^6) were then labelled with Annexin V and propidium iodide as described by the manufacturer. The labelled cells were analysed using a FACScalibur flow cytometer (BD Biosciences).

2.2. Conventional PCR and quantitative real-time PCR (qRT-PCR)

The mRNA was isolated using the Trizol method, according to the manufacturer's instructions. One microgram (1 μg) of total RNA was used for reverse transcription using a reverse transcription kit (Promega, USA). The resulting cDNA was then used in conventional PCR and qRT-PCR. Using SYBR Green with a Bio-Rad System (Bio-Rad, USA), the mRNA levels of target genes were measured, using the mRNA level of β-actin as an internal control. Conventional RT-PCR was performed as described previously (Senkal et al., 2007). The specific primer sets to perform qRT-PCR for β-actin, CerS4 and CerS5 were designed as follows: β-actin: forward 5′ gcgggaaatcgtgcgtgacatt 3′ and reverse 5′ gatggagttgaaggtagtttcgtg 3′; CerS4: forward 5′ ctctggtg ctgctgttac 3′ and reverse 5′ gactcgtagtatgtggtgtag 3′; and CerS5: forward 5′ atggtggctcctcaatgg 3′ and reverse 5′ aggtggtcacatcttcttcc 3′. The thermal conditions were run as follows: 95 °C for 2 min; 94 °C for 30 s; x* for 30 s; 72 °C for 1 min; repeat from step 2 for 39 cycles; 72 °C for 10 min; and 1 °C for 17 s. x* represents the annealing temperature (54 °C for β-actin; 53 °C for CerS4 and 55 °C for CerS5). The reactions were done in triplicate for at least three independent biological replicates. The Livak method ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008) was used to calculate the relative expression level of CerS4 and CerS5 in endometrial and colon cells. The GMMe and Caco-2 cell lines were used as calibrator samples, whereas Hec-1A and HT-29 were used as test samples. Furthermore, all untreated cells were used as calibrator samples while treated cells were used as test samples.

2.3. Statistical analysis

The results of each experiment were expressed as means ± standard error means (SEM) for at least three independent experiments, using GraphPad Prism 5. Significant differences were determined using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A probability level of $P < 0.05$ was considered significant.

2.4. Bio-informatics analysis

The use of bio-informatics to discover disease causing genes and how these genes and their products are regulated is growing. The amino acid sequences of CerS4 (NP_078828) and CerS5 (NP_671723) were obtained from NCBI (www.ncbi.nlm.niv.gov). These sequences were aligned using the Omega program to determine sequence similarity.

3. Results

3.1. Induction of apoptosis by anastrozole and 5-FU in endometrial and colon cells is more effective in cancerous (undifferentiated?) cell lines

To examine the apoptotic roles of anastrozole and 5-FU in endometrial and colon cells, FACS was performed. This was done using the Annexin V-FITC detection kit I (BD). For apoptosis analysis, cells were stained with fluorochromes, Annexin V-FITC and propidium iodide (PI). The cytograms in Fig. 1 illustrate the apoptosis percentage rate in GMMe and Hec-1A endometrial cells before and after treatment with anastrozole. This includes control samples with and without staining. The 24 h treatments show an increased apoptosis percentage rate, with Hec-1A cells demonstrating an ~2× increase in apoptosis percentage rate at 31.34% compared to 17.13% of GMMe cells as shown in Fig. 2. The apoptosis cytograms and apoptosis analysis bar graphs representing the response of Caco-2 and HT-29 colon cells to fluorouracil (5-FU) are illustrated in Figs. 3 and 4 respectively. The apoptotic response of these cells to 5-FU is somewhat delayed with very little apoptosis being observed at 24 h after treatment. The levels of apoptosis then increase significantly at 48 h after exposure. This is especially true in the HT-29 cells.