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Novel anti-apoptotic effect of Bcl-2: Prevention of polyamine depletion-induced cell death

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Abstract

The spermine analogue N^1,N^{11}-diethylnorspermine (DENSPM) efficiently depletes the polyamine pools in the breast cancer cell line L56Br-C1 and induces apoptotic cell death via the mitochondrial pathway. In this study, we have over-expressed the anti-apoptotic protein Bcl-2 in L56Br-C1 cells and investigated the effect of DENSPM treatment. DENSPM-induced cell death was significantly reduced in Bcl-2 over-expressing cells. Bcl-2 over-expression reduced DENSPM-induced release of the pro-apoptotic proteins AIF, cytochrome *c*, and Smac/DIABLO from the mitochondria. Bcl-2 over-expression reduced the DENSPM-induced activation of caspase-3. Bcl-2 over-expression also prevented DENSPM-induced Bax cleavage and reduction of Bcl-X_L and survivin levels. The DENSPM-induced activation of the polyamine catabolic enzyme spermidine/spermine N^1-acetyltransferase was reduced by Bcl-2 over-expression, partly preventing polyamine depletion. Thus, Bcl-2 over-expression prevented a number of DENSPM-induced apoptotic effects.

Keywords: Bcl-2; Apoptosis; Spermine analogue; Cytochrome *c*; Human breast cancer

1. Introduction

Apoptosis is a process of controlled cell deletion that plays a fundamental role in normal tissue development and homeostasis (Kerr et al., 1972; Song and Steller, 1999; Nagata, 2000). The apoptotic process is accompanied by cytoskeletal rearrangement, cell membrane disruption and blebbing, cell shrinkage, nuclear condensation, DNA fragmentation, and the formation of apoptotic bodies (Kerr et al., 1972; Song and Steller, 1999; Nagata, 2000). One of the most fundamental points in apoptosis is the activation of a family of proteases, i.e. caspases, which execute the cell death program (Earnshaw et al., 1999). The effector caspase caspase-3 is, e.g., activated when the apoptogenic factor cytochrome *c* is released into the cytosol from the mitochondria (Yang et al., 1997; Earnshaw et al., 1999).

The Bcl-2 family members can act as critical regulators of pathways involved in apoptosis, either as inhibitors or promoters of cell death signals (Reed, 1998). The inhibitors, i.e. anti-apoptotic proteins, include Bcl-2 and Bcl-X_L, whereas pro-apoptotic proteins, or promoters, include Bax, Bcl-X_S, and Bad (Reed, 1998; Fadeel et al., 1999; Harris and Thompson, 2000). One of the most studied negative regulators of apoptosis is the proto-oncogene *Bcl-2*. Bcl-2 over-expression prevents Bax cleavage and cytochrome *c* release (Yang et al., 1997; Susin et al., 1999). Other mitochondrial proteins involved in the regulation of the apoptotic machinery include the pro-apoptotic proteins Smac/DIABLO (Cregan et al., 2004; Saelens et al., 2004) and apoptosis inducing factor 1 (AIF-1 or AIF) (Susin et al., 1999; Cregan et al., 2004; Saelens et al., 2004). AIF plays an important role in the regulation of caspase-independent cell

Abbreviations: AIF, apoptosis inducing factor; DENSPM, N^1,N^{11}-diethylnorspermine; DEVD, amino acid sequence Asp-Glu-Val-Asp; ECL, enhanced chemiluminescence; HEB, hypotonic extraction buffer; HRP, horse radish peroxidase; PI, propidium iodide; *p*-NA, *p*-nitroanilide; PIPES, 2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid; PPAR-γ, peroxisome-proliferator-activated receptor-γ; SSAT, spermidine/spermine N^1-acetyltransferase.

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death. It is known that Smac/DIABLO can initiate caspase activation but recently it has been suggested that Smac/DIABLO is also potent in provoking caspase-independent cell death (Cregan et al., 2004). Survivin is a member of the inhibitor of apoptosis protein family that has been implicated in both control of cell division and inhibition of apoptosis. The anti-apoptotic function of survivin appears to be related to the ability to both directly and indirectly inhibit caspases (Shankar et al., 2001).

Bcl-2 also interacts with K-Ras (Spear et al., 1998; Rebollo et al., 1999; Denis et al., 2003). K-Ras belongs to a family of small monomeric GTPases that are known to play a key role in the signalling pathways which control cell survival, differentiation, migration, and proliferation (Philips, 2005). K-Ras indirectly affects the expression of the polyamine catabolic enzyme spermidine/spermine-N^1-acetyltransferase (SSAT) by binding the transcription factor peroxisome-proliferator-activated receptor-γ (PPAR-γ). PPAR-γ is a transcription factor for SSAT and thus K-Ras can inhibit activation of SSAT (Ignatenko et al., 2004).

In all eukaryotic cells, the polyamines putrescine, spermidine, and spermine are essential for cell proliferation, cell differentiation, and viability (Thomas and Thomas, 2001). Intracellular levels of polyamines are highly regulated via biosynthesis, catabolism, uptake, and excretion. Non-proliferating cells have low polyamine levels and growth stimulation requires an increase in the polyamine pools through activation of the biosynthetic enzymes (Thomas and Thomas, 2001; Oredsson, 2003). Polyamine pool depletion is mainly achieved by inhibition of the biosynthetic enzymes and activation of catabolism (Seiler, 2003a,b). There are numerous compounds with different mechanisms of action. The polyamine analogues efficiently deplete the cellular polyamines but cannot take over the role of the natural polyamines (Pegg et al., 1989; Fogel-Petrovic et al., 1997). One of these analogues is the symmetrical spermine analogue N^1,N^{11}-diethylnorspermine (DENSPM). Inside the cells, DENSPM induces a rapid depletion of all polyamines due to down-regulation of biosynthetic enzymes and up-regulation of the catabolic enzyme SSAT (Pegg et al., 1989; Fogel-Petrovic et al., 1997).

We have previously shown that the human breast cancer cell line L56Br-C1 is highly sensitive to DENSPM treatment, which induces apoptosis via the mitochondrial pathway (Hegardt et al., 2002; Holst and Oredsson, 2005). In this paper, we have transfected L56Br-C1 cells with Bcl-2 and present data showing that high expression of Bcl-2 can prevent DENSPM-induced apoptosis by various mechanisms.

2. Material and methods

2.1. Materials and reagents

Cell culture medium components were purchased from Biochrom, Berlin, Germany. Tissue culture plastics were purchased from Nunc, Roskilde, Denmark. The mammalian expression vector pcDNA3, carrying cDNA for Bcl-2, was kindly provided by Professor Urban Gullberg (Department of Haematology, Lund University, Lund, Sweden). Antibodies against AIF-1 (551429), Bad (610391), Bax (554104), Bcl-2 (556354), Bcl-X_L (551020), cytochrome *c* (556433), and Smac/DIABLO (612245) were purchased from BD Bioscience, San Diego, CA, USA. The antibody against β-actin was purchased from Abcam, Cambridge, UK. The antibody against caspase-3 (AHZ0052) was purchased from Biosource, Camarillo, CA, USA, and the antibody against survivin (sc-17779) was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Horseradish peroxidase (HRP)-conjugated secondary antibodies used for all proteins for Western blot analysis were purchased from DAKO Cytomation Denmark A/S, Glostrup, Denmark. Hybond enhanced chemiluminescence (ECL®) nitrocellulose membrane and Advanced ECL® protein detection reagent were purchased from Amersham Biosciences Ltd., Buckinghamshire, UK. Pre-cast NuPage® Novex 4–12% Bis-Tris SDS polyacrylamide gels (thickness of 1.0 mm, 15 wells) were purchased from Invitrogen Life Technologies, Stockholm, Sweden. Phosphate-buffered saline (PBS: 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, 0.2 g/L KH_2PO_4, pH 7.3) was purchased from Oxoid Ltd., Basingstoke, Hampshire, UK. DENSPM was purchased from Tocris Cookson Ltd., Bristol, UK. DENSPM was dissolved in PBS to give a stock solution of 2 mM. The stock solution was sterile-filtered and stored at −20 °C. [^{14}C]Acetyl coenzyme A was purchased from Sigma Chemical Co., St. Louis, MO, USA. CPP32/Caspase-3 colorimetric protease assay kit was purchased from Medical & Biological Laboratories Co. Ltd., Nagoya, Japan. Gene Pulser® Cuvette (0.4 cm) was purchased from Bio-Rad, Hercules, CA, USA. Geneticin was purchased from Invitrogen, Carlsbad, CA, USA. Propidium iodide (PI) was purchased from ICN Biomedicals Inc., Irvine, CA, USA. Protease inhibitor cocktail set III was purchased from Calbiochem, San Diego, CA, USA. Ribonuclease A type II (RNase A) was purchased from Sigma Chemical Co. The L56Br-C1 cell line was established in Lund, Sweden (Johannsson et al., 2003).

2.2. Cell culture

The human breast cancer cell line L56Br-C1 was cultured at 37 °C in a humidified incubator with 5% CO_2 in air. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum, non-essential amino acids, insulin (10 μg/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Johannsson et al., 2003; Holst and Oredsson, 2005; Holst et al., 2006). After isolation of stable transfectants, as described below, the cells were maintained in cell culture medium supplemented with 25 μg/ml geneticin. Cells were seeded in the absence or presence of 1 or 10 μM DENSPM. Both detached and attached cells were harvested, the latter by trypsinisation, and pooled (detached + attached cells) after 24 and 48 h of treatment. After counting in a haemocytometer, the cells were pelleted at 900 × *g* for 10 min at 4 °C and handled for further analysis as described below. Only opalescent cells were counted. These cells excluded the vital stain trypan blue.

2.3. Over-expression of Bcl-2

In the pcDNA3 vector, the expression of the inserted *Bcl-2* gene is driven by the cytomegalovirus promoter (Chylicki et al., 2000). The vector also carries an ampicillin resistance gene for bacterial selection and a neomycin resistance gene for mammalian cell selection. Prior to the transfection procedure, pcDNA3/Bcl-2 was linearised by endonuclease cleavage at ScaI sites. Control cells were transfected with the linearised empty vector (pcDNA3). Exponentially growing L56Br-C1 cells (8×10^6) were pelleted, re-suspended in 500 μl fresh cell culture medium at 37 °C. Plasmid DNA (15 μg) in 300 μl sterile PBS was added to the cell suspension and the sample was then transferred to a 0.4 cm Gene Pulser® Cuvette. The DNA was introduced into the cells by electroporation using a Gene Pulser® II (Bio-Rad). The cells were pulsed with 300 V at 250 μF. After a recovery period of 5 min on ice, the cells were seeded in fresh cell culture medium. Geneticin (0.5 mg/ml) was added after 3 days for the selection of stably transfected cells. L56Br-C1 cells transfected with the empty vector were named L56Br/pcDNA3 and L56Br-C1 cells transfected with the pcDNA3/Bcl-2 vector were named L56Br/Bcl-2.

2.4. Flow cytometry and data analysis

Cells were re-suspended in ice-cold 70% ethanol and stored at −20 °C until analysis. Nuclear DNA was stained with PI-nuclear isolation medium (PBS containing 100 μg/ml PI, 0.60% Nonidet P-40 (NP-40) and 100 μg/ml