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Bacillus intermedius ribonuclease (BINASE) induces apoptosis in human ovarian cancer cells

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ABSTRACT

The cytotoxic effects of *Bacillus intermedius* RNase (binase) towards ovarian cancer cells (SKOV3 and OVCAR5) were studied in comparison to normal ovarian epithelial cells (HOSE1 and HOSE2). Binase decreased viability and induced the selective apoptosis of ovarian cancer cells. The apoptosis rate was 50% in SKOV3 and 48% in OVCAR5 cells after 24 h of binase treatment (50 μg/ml). Binase-induced apoptosis in these cell lines was accompanied by caspase-3 activation and poly(ADP-ribose) polymerase fragmentation. Normal ovarian epithelial cells were not affected by binase, except for a slight decrease of HOSE2 cell viability and the appearance of traces of activated caspase-3, but not the poly(ADP-ribose) polymerase 85-kDA fragment. Binase did not induce alteration of EZH2 (enhancer of zeste-homolog-2) protein expression neither, in tumor nor in normal cells. In conclusion, selective binase-induced cell death and apoptosis via poly(ADP-ribose) polymerase fragmentation may serve as a new treatment option against ovarian cancer progression.

1. Introduction

Ovarian cancer affects approximately 1 in 70 women during their lifetime and is regarded as the most lethal gynecologic malignancy (Krylova et al., 2006). It takes 7th place in the list of age-standardized cancer mortality rates for female population in the world (Ferlay et al., 2013) and 5th place in the USA (Howlader et al., 2012). More than 90% of ovarian cancers are classified of epithelial origin. Various targeted therapeutics – including monoclonal antibodies against growth factor receptors, inhibitors of tyrosine kinase, poly(ADP-ribose) polymerase (PARP) and angiokinase – have been explored for ovarian cancer therapy (Campos and Ghosh, 2010). A novel principle in anticancer therapy is based on destroying tumor cell RNA by exogenous cytotoxic ribonucleases (RNases) (Ardelt et al., 2003; Cabrera-Fuentes et al., 2012; Fischer et al., 2014, 2013; Leland and Raines, 2001; Libonati et al., 2008). Some cytotoxic RNases of bacterial nature, such as *Bacillus intermedius* – binase (Cabrera-Fuentes et al., 2013; Makarov and Ilinskaya, 2003; Makarov et al., 2008), *Bacillus amyloliquefaciens* – barnase (Edelweiss et al., 2008; Ulyanova et al., 2011) and *Streptomyces aureofaciens* RNase Sa3 (Sevcik et al., 2002), are not inhibited by mammalian ribonuclease inhibitor and may selectively induce apoptosis of different tumor cell lines.

Binase is a well-characterized, highly cationic RNase that hydrolyzes RNA molecules, predominantly after purine (guanine) residues, with formation of 3′-mono- and 3′-

Abbreviations: Binase, *Bacillus intermedius* RNase; EOC, Epithelial ovarian cancer; EZH2, Enhancer of zeste homolog 2 protein; PARP, Poly(ADP-ribose) polymerase; RNase, Ribonuclease.

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dinucleotides as final products. The reaction does not require any co-factors and reaction products may induce various biological effects (Makarov et al., 2008; Ulyanova et al., 2011). Previously, we have shown that binase selectively inhibited the growth of cells expressing oncogenes like *ras* (Ilinskaya et al., 2001), *kit* or *AML-ETO or FLT3-ITD* (Ilinskaya et al., 2007; Mit'kevich et al., 2013; Mitkevich et al., 2011), but binase did not affect proliferation of v-*fms*- or v-*src*-transformed fibroblasts (Ilinskaya et al., 2001). Investigations aiming to find certain oncogenes that could affect the sensitivity of malignant cells towards RNases are important for future development of RNase-based therapeutics.

The main oncogenes that have been implicated in ovarian cancer include *HER-2, myc, ras* and *p53* (Berchuck et al., 1992). It is known that *p53* is inactivated in virtually all high-grade serous ovarian cancers (Cancer Genome Atlas Research, 2011). We previously found that the quantity of proapoptotic *p53* mRNA increased during binase treatment and this contributed to apoptosis of tumor cells (Mitkevich et al., 2010). Based on the known susceptibility of cells expressing oncogenic *ras* (Ilinskaya et al., 2001) and *p53* (Mitkevich et al., 2010) to binase, we propose that the oncogene expression profile in epithelial ovarian cancer (EOC) cells might contribute to induction of their selective killing by binase.

Recently, anticancer effects of binase were confirmed *in vivo* using a number of animals models. Binase administration at a dose range of 0.1–5 mg/kg results in retardation of primary tumor growth and inhibition of metastasis formation (Mironova et al., 2013). Binase caused a decrease of the hepatotoxicity for polychemotherapy, while maintaining its antitumor effect (Mironova et al., 2013) as well as, reduction of destructive changes in the liver caused by tumor development, and recovery of its regenerative potential (Sen'kova et al., 2014). Additionally, binase did not induce apoptosis in leukocytes of healthy donors and in normal myeloid progenitor cells and had no superantigenic properties (Ilinskaya et al., 2007).

The aim of this work was to evaluate the effects of binase on two EOC cell lines (SKOV3, OVCAR5) in comparison to two normal human ovarian epithelial cell isolates (HOSE1, HOSE2) and to assess whether or not the binase cytotoxic effect is selective. Additionally, we determined the expression of common apoptotic marker proteins and EZH2 (enhancer of zeste-homolog-2), which silences gene expression through trimethylating lysine 27 residue of histone H3 and is often overexpressed in ovarian cancer (Li et al., 2010). We previously found that EZH2 knockdown induced apoptosis in human EOC cells (Garipov et al., 2013). Our new data provide strong evidence that selective binase-induced cell death and apoptosis via poly(ADP-ribose) polymerase fragmentation may serve as a new treatment option against ovarian cancer progression.

2. Materials and methods

2.1. Enzyme

Binase was isolated from *B. intermedius* culture fluid as a homogeneous single-chain 12.3-kDa protein that consist of 109 amino acid residues. The enzyme purification was carried out by a previously described procedure (Makarov et al., 1993), and enzyme purity was confirmed by electrophoresis (Cabrera-Fuentes et al., 2013). Binase is a highly cationic guanyl-specific RNase with pI value of 9.5 that catalyzes cleavage of RNA without a need for metal ions and cofactors (Makarov et al., 1993). Catalytic activity of binase was 1.4×10^7 U/mg when measured against high molecular weight RNA from yeast (Ilinskaya et al., 1996). One unit is the amount of enzyme that increases the extinction at 260 nm of acid-soluble products of RNA hydrolysis by one unit per min measured at 37 °C and pH 8.5.

2.2. Cell cultures

Two EOC cell lines (SKOV3 and OVCAR5) and two cultures of primary human ovarian epithelial cells (HOSE1 and HOSE2) were used. Primary HOSE cells were isolated as previously described (Bellacosa et al., 2010). Ovary specimens were kindly provided by Tatarstan Regional Clinical Cancer Center (TRCCC). All procedures were performed in accordance with the legal requirements of the Ministry of Health of the Republic of Tatarstan, Russian Federation and were approved by the Research Ethics Committee of Kazan Medical University and TRCCC, Russia (Protocol № 4/07.05.2009); all patients gave written informed consent.

EOC cells were grown in RPMI-1640 medium (Biochrom KG Berlin, Germany) supplemented with 10% fetal calf serum (Sigma, Taufkirchen, Germany), 2 mM glutamine and antibiotics (penicillin and streptomycin, 100 U/ml each). HOSE cells were grown in DMEM medium with the same supplements, except for the content of fetal calf serum (4%) and additional ITS media supplement (Sigma, Taufkirchen, Germany). All cells were maintained at 37 °C in a CO_2 incubator in a humid atmosphere with 5% CO_2.

2.3. Viability

Cellular viability was assessed using a WST-1-based test (Roche Diagnostics GmbH, Mannheim, Germany). This test is based on cleavage of a water-soluble tetrazolium salt by mitochondrial dehydrogenases. The cells were plated into 96-well plates (3×10^3 cells per well) and cultured at 37 °C. After 12 h, when cells were attached, binase (10, 50 and 150 μg/ml) was added and cells were incubated for additional 24 h. Next, medium was changed and cells were incubated with the WST-1 reagent for 120 min at 37 °C. The absorbance was measured on an Anthos 2020 microplate reader (Anthos Labtech Instruments GmbH, Wals, Austria) at 440 nm, the reference wavelength was 620 nm. A mixture of cell-free medium with WST-1 reagent was used as a background control. Viability of untreated cells was set as 100%.

2.4. Cytometry

Apoptosis was measured using a Guava easyCyte™ (Millipore) flow cytometer and Guava Nexin reagent (4500-0455, Millipore). Guava Nexin (125 μl) was added to 100 μl of cell suspension containing 2×10^5 cells/ml and incubated for 20 min in the dark before analysis. Four cell lines

that were treated or left untreated with different concentrations of binase (10–150 μg/ml) were tested.

2.5. Immunoblotting

SKOV3, OVCAR5, HOSE1 and HOSE2 cells were treated for 24 h with binase (10, 50 and 150 μg/ml). Total cell lysates were obtained using RIPA buffer (Millipore, Billerica, MA) supplemented with complete ULTRA proteinase cocktail (Roche Diagnostics GmbH, Mannheim, Germany), 0.1 mM phenylmethylsulfonyl fluoride (Sigma, Taufkirchen, Germany) and 1 mM sodium vanadate (Sigma, Taufkirchen, Germany). The antibodies used for immunoblotting analysis were from the indicated suppliers as following: anti-cleaved-PARP p85 fragment (Promega, Madison, WI), anti-cleaved-caspase-3 (Cell Signaling Technologies, Beverly, MA), anti-β-actin (Cell Signaling Technologies, Beverly, MA), anti-EZH2 (BD Biosciences, San Jose, CA) and anti-H3K27Me3 (Millipore, Billerica, MA).

2.6. Statistics

Data were analyzed by nonparametric Mann–Whitney test and One-way ANOVA followed by Bonferroni's multiple comparisons test, when appropriate, to determine statistical significance of the data differences using GraphPad Prism version 6.00. Differences with $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Binase reduces viability of ovarian cancer cells

The viability of HOSE1 cells in the presence of 10 μg/ml binase was comparable to cells grown in the absence of binase, while the viability of HOSE2 cells was slightly decreased by 28 ± 12% ($P = 0.0420$). In contrast epithelial ovarian cancer cells were much more sensitive to the cytotoxic action of binase, especially SKOV3 cells. Their viability decreased to less than 40% after 24 h of treatment with binase at lowest concentration (10 μg/ml; Fig. 1A). Similarly, OVCAR5 cell line viability was reduced by 25% at the same binase concentration. Higher concentrations of binase induced concentration-dependent inhibition of EOC cell growth. The viability of SKOV3 and OVCAR5 cells treated with high concentrations of binase (150 μg/ml) was 17 ± 1% ($P < 0.0001$) and 28 ± 5% ($P = 0.0062$), respectively. The same binase concentration caused a smaller decrease in viability of non-tumorigenic ovarian epithelial cells (the viability of HOSE1 was 82 ± 8%, $P = 0.1240$; HOSE2 - 72 ± 11%, $P = 0.0164$; Fig. 1A).

3.2. Binase induces apoptosis in ovarian cancer cells

The cytometric distribution of ovarian cells after cultivation for 24 h with 10 μg/ml binase (the lowest concentration used) revealed that the enzyme caused apoptosis of 22 ± 2% (SKOV3, $P = 0.0060$) and 16 ± 3% (OVCAR5, $P = 0.0011$) of the respective cell population. For HOSE1 and HOSE2 cells, the number of apoptotic cells did not exceed 7% (Fig. 1B). The fraction of apoptotic cells became

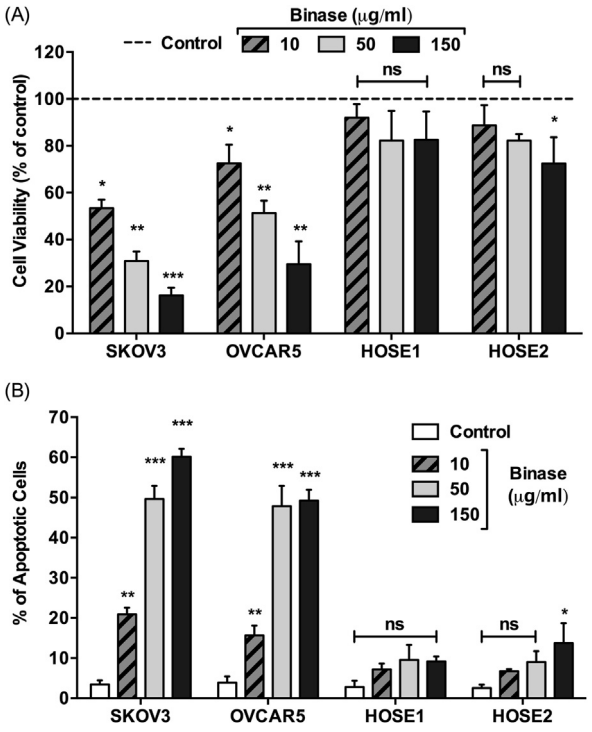

Fig. 1. Viability (A) and apoptosis (B) of ovarian epithelial cells treated for 24 h with binase at concentrations ranging from 10 to 150 μg/ml. Values represent mean ± SD ($n = 6$); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, ns = non-significant. (A) Viability was assessed with a WST-1-based test, based on cleavage of a water-soluble tetrazolium salt by mitochondrial dehydrogenases. ROD – relative optical density measured at 440 and 620 nm. (B) Apoptosis was detected cytometrically with the Guava Nexin staining kit.

larger as the binase concentration was increased and reached 60 ± 3% (SKOV3; $P < 0.0001$) and 49 ± 4% (OVCAR5; $P < 0.0001$) at the maximal concentration of the enzyme, as compared to 4 ± 1% without binase (Fig. 1B). A slight increase of the apoptotic cell fraction was observed in the populations of HOSE1 and HOSE2 cells treated with 150 μg/ml binase (12 ± 2%, $P = 0.0328$ and 22 ± 1%, $P = 0.0306$; respectively) (Fig. 1B).

3.3. Binase induces activation of caspase-3 but does not affect EZH2 expression

Western blot analysis showed induction of apoptosis in SKOV3 and OVCAR5 cells treated with binase. Activation of caspase-3 was detected in both cancer cell lines after exposure to binase at 50 μg/ml for 24 h (Fig. 2A). Small amounts of activated caspase-3 were detected in normal ovarian epithelial cells, HOSE1 and HOSE2, which was not followed by PARP fragmentation. In contrast, activation of caspase-3 in EOC cells was accompanied by PARP fragmentation; this fragmentation did not occur in normal cells (Fig. 2A).

Binase did not reduce the expression of EZH2 protein and did not change the levels of trimethylated lysine 27 residue of histone H3 in EOC cell lines (Fig. 2B).

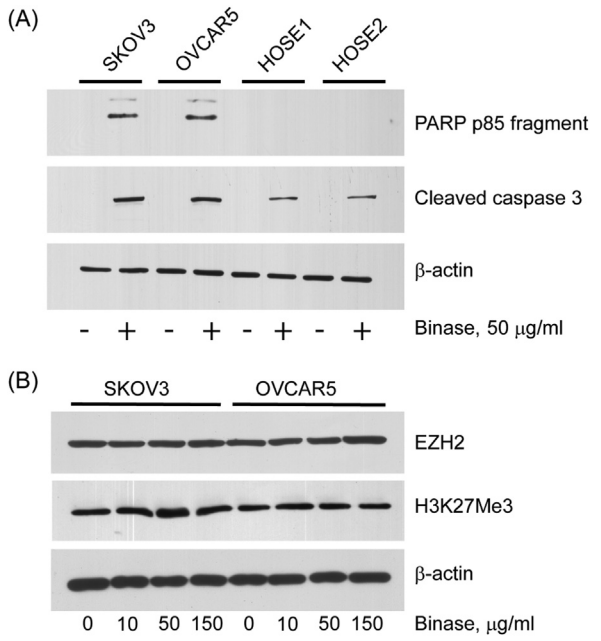

Fig. 2. (A) Detection of apoptotic markers in EOC cells (SKOV3, OVCAR5) compared to normal ovarian epithelial cells (HOSE-1, HOSE-2) after binase treatment (24 h, 50 μg/ml). (B) Analysis of EZH2 expression and H3K27Me3 levels in SKOV3 and OVCAR5 cells treated with binase at different concentrations (10, 50 and 150 μg/ml) for 24 h. β-actin was used as a reference protein. Representative images of multiple experiments are shown ($n = 6$).

4. Discussion

Ovarian cancer is difficult to detect and typically presents at an advanced stage. Despite the sensitivity to induction of chemotherapy, the majority of patients develop recurrent disease, and for this reason, ovarian cancer is among the most lethal of all cancers (Cancer Genome Atlas Research, 2011). Here, we studied the cytotoxicity of binase towards EOC cells in comparison to its action on normal epithelial cells to obtain comparative data supporting the future development of new anticancer agents based on microbial RNases.

We have shown high sensitivity of SKOV3 and OVCAR5 cell lines to binase. Increased binase concentrations induced an increase in cytotoxicity (Fig. 1A) and apoptosis in cancer cell lines (Fig. 1B), but did hardly affect normal cells, indicating considerable selectivity of this RNase. SKOV3 cells were especially sensitive to binase (IC_{50} ~0.8 μM). This value is comparable to that for onconase, a well-studied cytotoxic RNase, towards NCI/ADR-RES cells (IC_{50} = 1.1 μM) (Castro et al., 2011), derived from the ovarian cancer cell line OVCAR-8 (Garraway et al., 2005). Moreover, the apoptotic fraction of the SKOV3 population reached 60% after 24 h of binase treatment, while for onconase-treated NCI/ADR-RES cell line this value was 9.1% (Castro et al., 2011). Compared to barnase, which was also cytotoxic (IC_{50} = 1.8 μM) and activated caspase-3 in SKOV3 cells (Edelweiss et al., 2008), binase was twice as effective. Sensitivity of OVCAR5 cells to binase in our experiments was substantially lower (IC_{50} = 4.0 μM; Fig. 1A). Reasons for this difference may include variable internalization of RNase into cells (Cabrera-Fuentes et al., 2013; Lee and Raines, 2008), different expression of RNase-cleaved microRNAs (Qiao et al., 2012), and/or different profiles of expressed oncogenes (Mitkevich et al., 2011).

Previously we have shown that cells expressing oncogenes such as v-*ras* or *p53* were susceptible to the apoptogenic action of binase (Ilinskaya et al., 2001; Mitkevich et al., 2010). Alterations in *p53* are a common event in advanced EOC (Soussi and Beroud, 2001). Mutations in *p53* are associated with a short-term survival benefit (Havrilesky et al., 2003). Mutations in the H-*Ras* gene, in contrast to Ki-*Ras*, have rarely been found in ovarian tumors, but physiologically activated H-Ras protein is commonly detected in human EOC cells (Rosen et al., 2006). Although the *ras* and *p53* mutation status for SKOV3 and OVCAR5 cell lines is inconclusive (Berglind et al., 2008), there are some reports certifying that SKOV3 cells are p53 negative (Shoshani et al., 2002) and do not express mutated Ras (Ihle et al., 2009), while OVCAR cells harbor significant amounts of Ras (Beiner et al., 2006) and mutant p53 (Meijer et al., 2013). Thus, we can assume that the sensitivity of EOC cells to binase may only partially based on the expression of these oncogenes.

Apoptosis in both SKOV3 and OVCAR5 cells detected cytometrically was confirmed by immunoblot detection of cleaved active form of caspase-3 (Fig. 2A), which is an effector caspase of both extrinsic and intrinsic apoptotic pathways. Previously, it was shown that binase activated both apoptotic pathways in Kasumi-1 cells; whereby the extrinsic apoptotic pathway is supported by activation of caspase-8, and the intrinsic apoptotic pathway activation increases Ca^{2+}, induced by the opening of mitochondrial permeability transition pores. However, Mitkevich et al. (2013) demonstrates also an absence of activation of caspase 9, the initiator caspase of the typical intrinsic (mitochondrial) apoptotic pathway.

We also observed fragmentation of the PARP protein, a well-known target for a number of enzymes including caspases in SKOV3 and OVCAR5 cells but not in normal ovarian cells (Fig. 2A). This data further indicates an activation of caspase-3 in EOC cells treated with binase. Here the product of caspase-3 activity, the 85-kDa PARP fragment, was detected by Western blot analysis (Fig. 2A), whereas, the PARP fragmentation was not observed in binase-treated HOSE1 or HOSE2 cells despite some activation of caspase-3 (Fig. 2A). This initial activation probably occurred in the early apoptotic phase of the cell population, which contributed to a slight decrease in the viability of normal cells (Fig. 1A) and consists of less than 25% of cells treated with the highest binase concentration (Fig. 1B). It is known that PARP inhibition contributes to the selective killing of breast cancer (BRCA)-deficient cancer cells (Lord and Ashworth, 2008). Though both SKOV3 and OVCAR5 cells carry the wild-type BRCA1 and BRCA2 (Stordal et al., 2013), PARP cleavage in these cells could be considered as an additional positive indicator of binase's antitumor effects.

We also tried to determine the influence of binase on EZH2, the catalytic subunit of histone methyltransferase (polycomb repressive complex 2), which trimethylates the K27 residue of histone H3 (H3K27Me3) and performed

target gene silencing (Li et al., 2010). EZH2 is known to play a key role in the maintenance of a drug-resistant subpopulation of cells in ovarian cancers undergoing chemotherapy (Rizzo et al., 2011). Moreover, knockdown of EZH2 induced apoptosis in human EOC cell lines (Garipov et al., 2013). EZH2 expression is abnormally elevated in cancer tissues compared to corresponding normal tissues, and high EZH2 levels correlate with advanced stages of disease and a poor prognosis (Shen et al., 2013). This is the first attempt to find an effect of exogenous RNase on one of the enzymes affecting epigenetic regulation of gene expression. Here, we reveal that the EZH2 protein expression and the level of a marker of its activity (H3K27Me3) did not change in binase-treated EOC cell lines (Fig. 2B). This means that the mechanism(s) of binase cytotoxic action did not involve cleavage or expression changes of EZH2. Nevertheless, the contributing mediators or the underling mechanisms remain unknown at present, and epigenetic markers might remain as potential therapeutic targets.

Despite all the progress in elucidation of the anticancer effects of RNases, the precise molecular mechanism of their antitumor effects still remains an area of future research. Nevertheless, our results indicate that binase might be an effective agent for EOC therapy.

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Conflict of interest

None of the authors has any conflicts of interest.

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