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Bovine seminal ribonuclease triggers Beclin1-mediated autophagic cell death in pancreatic cancer cells

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ABSTRACT

Among the large number of variants belonging to the pancreatic-type secretory ribonuclease (RNase) superfamily, bovine pancreatic ribonuclease (RNase A) is the proto-type and bovine seminal RNase (BS-RNase) represents the unique natively dimeric member. In the present manuscript, we evaluate the anti-tumoral property of these RNases in pancreatic adenocarcinoma cell lines and in nontumorigenic cells as normal control. We demonstrate that BS-RNase stimulates a strong anti-proliferative and pro-apoptotic effect in cancer cells, while RNase A is largely ineffective. Notably, we reveal for the first time that BS-RNase triggers Beclin1-mediated autophagic cancer cell death, providing evidences that high proliferation rate of cancer cells may render them more susceptible to autophagy by BS-RNase treatment. Notably, to improve the autophagic response of cancer cells to BS-RNase we used two different strategies: the more basic (as compared to WT enzyme) G38K mutant of BS-RNase, known to interact more strongly than wt with the acidic membrane of cancer cells, or BS-RNase oligomerization (tetramerization or formation of larger oligomers). Both mutant BS-RNase and BS-RNase oligomers potentiated autophagic cell death as compared to WT native dimer of BS-RNase, while the various RNase A oligomers remained completely ineffective. Altogether, our results shed more light on the mechanisms lying at the basis of BS-RNase antiproliferative effect in cancer cells, and support its potential use to develop new anti-cancer strategies.

1. Introduction

Pancreatic adenocarcinoma is one of the most aggressive and devastating human malignancies and the fourth leading cause of worldwide cancer-related deaths. Standard treatments for advanced disease include monotherapy with gemcitabine (2′,2′-difluoro-2′-deoxycytidine), which has, however, a response rate of less than 20% [1]. Therefore, during the last few years, the identification of valuable targets [2,4] and novel efficient therapeutic strategies against pancreatic adenocarcinoma has been extensively investigated [5,7]. Among the biological events associated with the response of cancer cells to therapeutics [8], autophagy is acquiring a growing interest. It is a highly conserved cellular process in which cytoplasmic materials, including organelles, are sequestered into double-membrane vesicles called autophagosomes and delivered to lysosomes for degradation or recycling. Besides its cytoprotective role in cellular homeostasis, autophagy can be a form of programmed cell death, designated as "autophagic cell death" [9]. Physiologically, autophagy has the role to preserve the balance between organelle biogenesis, protein synthesis and their clearance [10]. More recently, autophagy has been shown to be an important mediator of pathological responses and to be engaged in the cross-talk with reactive oxygen species involved in cell signaling and protein damage [11]. Interest in the role of autophagy in cancer research starts from the discovery that BECN1 (the gene encoding the pro-autophagic protein Beclin1, also known as ATG6) is also a tumor suppressor gene [12], revealing that autophagy is under the control of a large panel of oncogenes and products of tumor suppressor genes [13]. The link between autophagy and antitumor activity prompted us to investigate the possible role of ribonucleases in this process. It is well known, in fact, that many ribonucleases display, besides their enzymatic activity, other remarkable biological activities [14,15], among which emerges a relevant cytotoxic effect against tumor cells *in vitro* and *in vivo* [16,19]. These findings aroused new interest in the fields of potential anticancer therapy related to this class of enzymes. In this work, we focused our attention in particular on two variants belonging to the secretory pancreatic-type RNase super-family: the proto-type bovine pancreatic ribonuclease (RNase A), which is monomeric in its native state, and the

Abbreviations: RNase, ribonuclease; BS-RNase, bovine seminal ribonuclease; WT, wild-type; CQ, chloroquine diphosphate; 3MA, 3-methyladenine; FBS, fetal bovine serum; SDS, sodium dodecyl sulphate; MDC, monodansylcadaverine; siRNA, small interfering RNA; D, dimers; T, trimers; TT, tetramers; LO, larger oligomers; RI, RNase inhibitor

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bovine seminal RNase (BS-RNase), a natively dimeric protein constituted by two identical subunits covalently bound through two antiparallel disulfide bridges. Each BS-RNase subunit shares 82% sequence identity and very close 3D structural and dynamical features in solution with RNase A [20]. In solution, BS-RNase is an equilibrium mixture containing 70% of N-terminal-swapped [21] and 30% of unswapped isoforms, respectively called MxM and M=M [22]. Notably, swapped MxM BS-RNase evades RI because of its dimericity, which persists also under the cytosol reducing environment [24], and can consequently bear a remarkable antitumor action [25], while RNase A, like most monomeric RNases, is strongly bound and inactivated in mammalian cells by the RNase Inhibitor (RI) [23]. Anyway, native RNase A can be induced [26] to form various N- or C-terminal domain-swapped dimers [27,29], trimers [30], and larger oligomers [31,32], all reconstituting the active site [26,28] and augmenting the enzymatic activity against dsRNA with respect to the native monomer [33]. Despite depending on the tumor type tested [34,36], RNase A oligomers can also acquire cytotoxic activity both *in vitro* and *in vivo* [37]. Also BS-RNase is known to oligomerize through the 3D domain swapping of both N- and C-termini, and its multimers show to be enzymatically and biologically more active than the native dimer(s) [38]. Furthermore, BS-RNase activity can be increased also by mutations which augment its basicity and capability to interact with cell membranes, as it occurs for the G38K mutant [39] that has been tested in this work. Thus, in the present manuscript we investigated whether the two mentioned secretory RNases (*i.e.*, RNase A and BS-RNase), and their respective oligomeric aggregates, could display an efficient anti-proliferative activity against pancreatic adenocarcinoma cells, as compared to nontumorigenic cell lines. Furthermore, considering its relevant role in the cell life-balance, we explored if autophagy could have a role in the RNase-mediated cancer cell death.

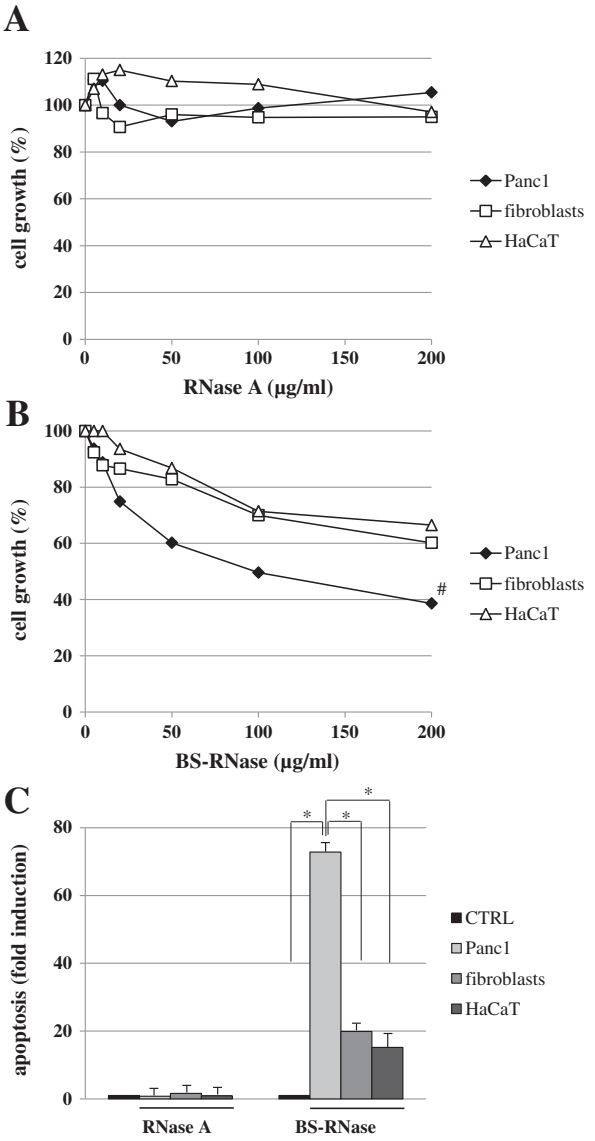

Fig. 1. Effect of RNase A and BS-RNase on growth inhibition and apoptosis in Panc1, fibroblasts, and HaCaT cells. (A and B) Cells were seeded in 96-well plates, incubated overnight, and treated with increasing concentrations of RNase A or BS-RNase for 72 h. Cell proliferation was determined using the Crystal Violet colorimetric assay. Values are the means of three independent experiments, each performed in triplicate. Statistical analysis: (#) $p < 0.05$ Panc1 vs fibroblasts or HaCaT. (C) Cells were seeded in 96-well plates, incubated overnight, and treated with increasing concentrations of RNase A or BS-RNase for 72 h. Apoptosis was analyzed using the annexinV/FITC binding assay. Values are the means (±SD) of three independent experiments, each performed in triplicate. Statistical analysis: (*) $p < 0.05$.

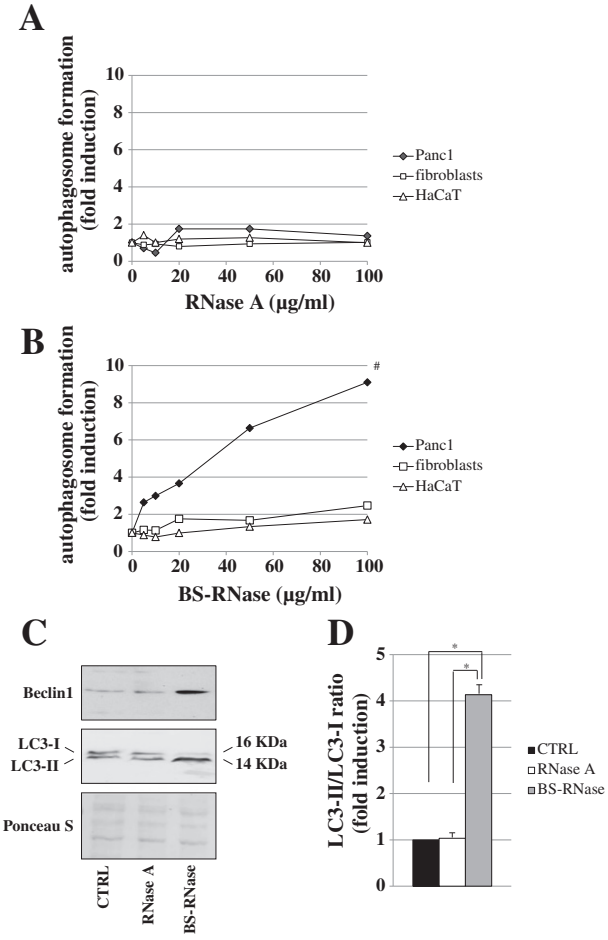

Fig. 2. Effect of RNase A and BS-RNase on autophagy in Panc1, fibroblasts, and HaCaT cells. (A and B) Cells were seeded in 96-well plates, incubated overnight, and treated with increasing concentrations of RNase A or BS-RNase for 72 h. Autophagosome formation assay was analyzed using the incorporation of MDC probe. Values are the means of three independent experiments, each performed in triplicate. Statistical analysis: (#) $p < 0.05$ Panc1 vs fibroblasts or HaCaT. (C) Cells were seeded in 60-mm diameter culture dishes, incubated overnight, and treated with 100 μg/ml RNase A or BS-RNase for 32 h. Whole-cell extracts were used for Western blot analysis of the autophagy-related proteins Beclin1 and LC3. Ponceau S staining was used as control loading. (D) Quantitative analyses of LC3-II/LC3-I ratio. The bands were scanned as digital peaks and the areas of the peaks were calculated in arbitrary units, as described in the "Materials and methods" section. The value of Ponceau S dye was used as normalizing factor. Values are the means of three independent experiments (±SD). Statistical analysis: (*) $p < 0.0$.