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Recombinant canine stromelysin 3 inhibits drug induced apoptosis *in vitro*

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ABSTRACT

Matrix metalloproteinases (MMPs) are zinc metalloproteases having a pivotal role in extracellular matrix degradation, tumor growth, apoptosis, angiogenesis, invasion, and development of metastases. Stromelysin 3 (ST3) increased tumor take by suppressing cell apoptosis. Our present goal was to set up an *in vitro* model in which we could study this new function of ST3. For this purpose, we analysed effect of vinblastine, an anti-cancer drug, on ST3 treated MDCK (Madin-Darby canine kidney) cells. We found a marked decrease in the percentage of vinblastine induced MDCK cell death when the cells were pre-treated with recombinant ST3 as assessed by FACS analysis. Our data confirmed and extended the anti-apoptotic function of ST3 *in-vitro*.

Key words: Apoptosis, FACS, Matrix metalloproteinases, Stromelysin 3, Vinblastine

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases which help in matrix degradation and tissue remodelling (Sternlicht and Werb 2001, Vu and Werb 2000). Expression and activity of these enzymes are regulated at different levels; they are expressed as zymogens, then processed to active forms after proteolytic cleavage and their activities are also controlled by a family of natural tissue inhibitors of metalloproteinases (TIMPs). An imbalance between MMPs and TIMPs is implicated in various physiological but also in pathological tissue remodelling processes, notably during cancer progression and metastasis (Brinckerhoff and Matrisian 2002, Egeblad and Werb 2002).

Stromelysin-3 (ST3, MMP–11) is a member with a unique pattern of expression and substrate specificity. ST3 was isolated from primary breast cancers and was overexpressed in virtually all invasive primary carcinomas (Anderson *et al.* 1995, Rouyer *et al.* 1994). ST3 is therefore proposed as a potential prognostic signature and also as an attractive target for therapeutic approaches directed against the stromal compartment of human carcinomas. Using murine models, it was demonstrated that ST3 expression was able to increase tumor take by suppressing cell apoptosis (Wu *et al.* 2001). Interestingly, normal ST3 expression during embryogenesis and tissue involution is associated with tissue remodelling and is also located in stromal cells in direct contact with apoptotic epithelial cells (Ishizuya-Oka *et al.* 2000, Damjanovski *et al.* 1999).

Earlier we heterologously expressed canine ST3 in *E. coli* and found that the recombinant protein possessed caseinolytic activity and facilitated migration of MDCK cells through BD Biocoat Matrigel invasion chambers (Sunil Kumar *et al.* 2013). In this paper we have analysed the effect of vinblastine on recombinant ST3 treated MDCK cells by flow cytometry.

MATERIALS AND METHODS

Recombinant canine ST3 (~52 kDa) was heterologously expressed in *E. coli* DH5a and purified to homogeneity by Ni-NTA affinity chromatography. MDCK cells were sub cultured in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml Streptomycin and 10% fetal calf serum and seeded in two 12 well plates at the concentration of 1×10^6 cells/ml. The plates were incubated at 37° C with 5% CO_2 in a CO_2 incubator for 18 h. At 65% confluency, rST3 and proteinase K (negative ST3 control) were added each to a final concentration of 50 nM. The plates were again incubated in the CO_2 incubator for 12 h. Now, vinblastine to a final concentration of 50 nM was added to the cells. Positive control consisted of cells treated with vinblastine only, while untreated cells served as blank. The plates were finally incubated for 48 h under the same conditions. After 48 h cells were harvested with trypsin versene solution and centrifuged at 3,000 rpm for 3 min to pellet the cells.

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Washing was done once with PBS and cell pellets were incubated in 200 µl of 1 × binding buffer mixed with 5 µl of annexin V-FITC solution for 15 min in dark at room temperature then centrifuged at 3,000 rpm for 3–4 min and finally cell pellet were incubated in 200 µl of 1 × binding buffer mixed with 10 µl propidium iodide solution in dark for 5 min and again centrifuged at 3,000 rpm for 4 min to remove unbound dye and pellet was resuspended in 200 µl 1 × binding buffer. Finally, 10,000 cells were analyzed per sample by BD FACS Calibur instrument.

Analysis of annexin V-FITC and propidium iodide (PI) staining by flow cytometry was done using FL1 as a signal detector for annexin V-FITC (for detecting apoptotic cells), FL2 for propidium iodide (for detecting necrotic cells). Samples were analyzed in "Cell quest" software of FACS Calibur. The positive cell counts were displayed in histogram stat. The difference in percent apoptosis and necrosis upon various treatments were statistically analysed by one way ANOVA using SPSS10 (Snedecor and Cochran 1994).

RESULTS AND DISCUSSION

The percent apoptosis and necrosis in MDCK cells determined by flow cytometric analysis upon different treatments are depicted in Figs. 1, 2 and Table 1. There were no visible changes in cells treated with rST3 alone. Vinblastine induced significant apoptosis (60.08±0.023%) in MDCK cells. However, the per cent apoptosis was significantly less (P≤0.05) in cells which were pre-treated with rST3 (1.29±0.023%). Proteinase K and vinblastine treatment caused significant necrosis (51.32±0.025%) as well as apoptosis (38.33±0.028%) in MDCK cells. The results suggested that vinblastine could not induce apoptosis in rST3 treated MDCK cells.

In both physiological and pathological conditions, many cells exhibit programmed cell death (PCD) with documented activation of an effector caspases. Nevertheless, there are also many exceptions in which other proteases (including cathepsins, MMPs, proteasomal proteases etc) display activation that can effectively trigger apoptosis (Lockshin and Zakeri 2004). MMPs regulate apoptosis in a context-dependent manner, in which the relative concentrations, tissue specificity, and spatio-temporal balance between MMPs and TIMPs also influence the proteolytic cascade, determining the commitment to PCD (Noel *et al.* 2012). In the present study, we found that

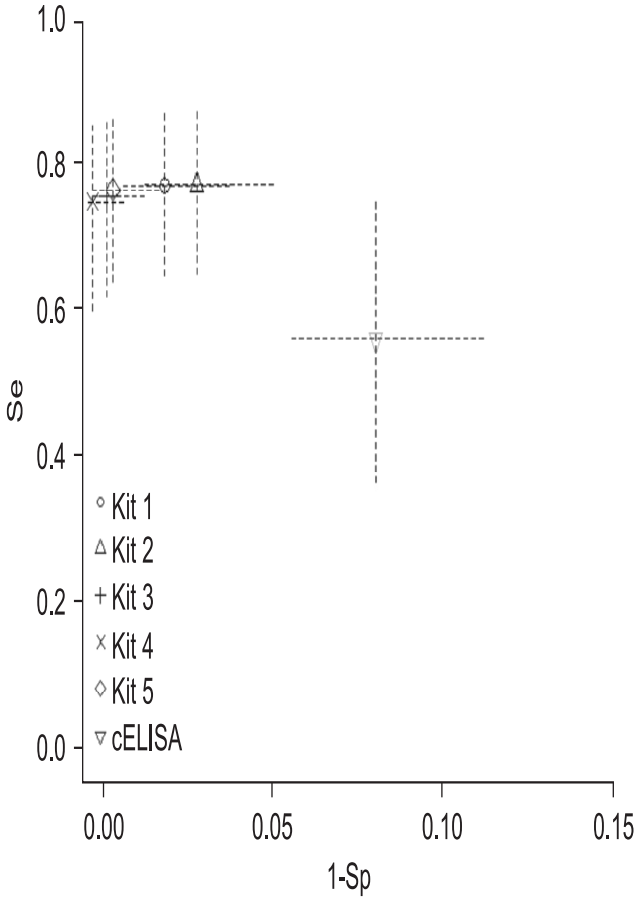

Fig.1. Histogram of FACS analysis of apoptosis in MDCK cells upon different treatments. (a) Untreated cells, (b) cells treated with rMMP11, (c) cells treated with vinblastine, (d) cells treated with rMMP11 and vinblastine, (e) cells treated with proteinase K (f) Cells treated with proteinase K, and vinblastine.

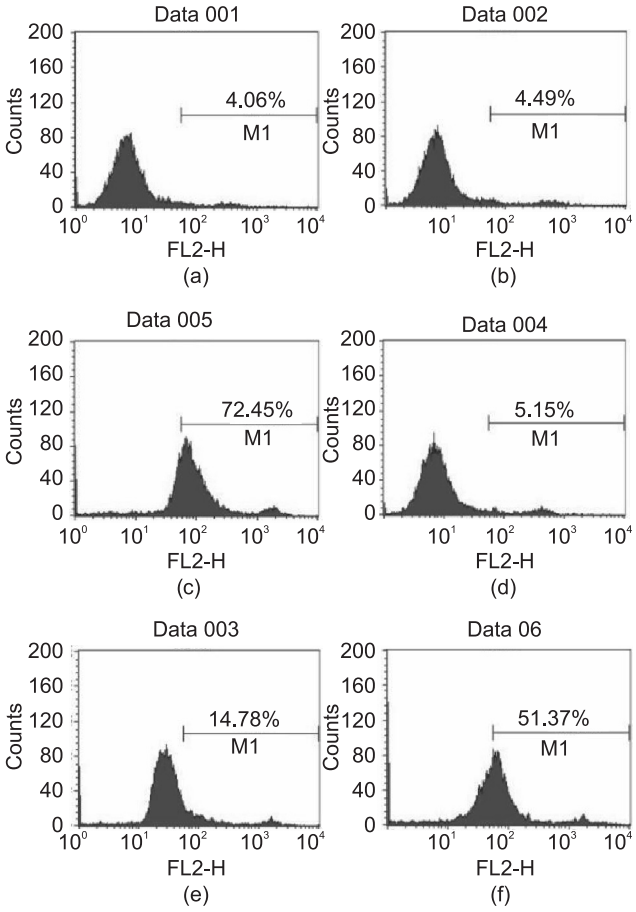

Fig.2. Histogram of FACS analysis of necrosis in MDCK cells upon different treatments. (a) Untreated cells, (b) cells treated with rMMP11, (c) cells treated with vinblastine, (d) cells treated with rMMP11 and vinblastine, (e) cells treated with proteinase K, (f) cells treated with proteinase K and vinblastine.

Table 1. Percent apoptosis and percent necrosis in MDCK cells upon different treatments

Cell treatment	% Apoptosis (FL1)	% Necrosis (FL2)
Cells alone	1.64^{d}±0.026	4.00^{d}±0.032
Cells + rST3	1.06^{d}±0.029	4.44^{d}±0.027
Cells + Vinblastin	60.08^{a}±0.023	72.40^{a}±0.026
Cells + Proteinase K	12.20^{c}±0.153	14.73^{c}±0.024
Cells + rST3 + Vinblastin	1.29^{d}±0.023	5.08^{d}±0.042
Cells + Proteinase K + Vinblastin	38.33^{b}±0.028	51.32^{b}±0.025

Values are mean±standard error. ^{abcd}Values differ significantly ($P<0.05$).

vinblastine (a potent apoptogenic agent) could not induce apoptosis in MDCK cells which were pre-treated with rST3. Previously, it was demonstrated that MMP-11 and MMP-7 (matrilysin) produced by tumor cells at early stages of cancer development cleaves CD95, reducing its surface expression and decreasing the CD95-mediated apoptosis of tumor cells; this proteolytic activity may contribute to an apoptosis-resistant phenotype in tumor cells, promoting immune-escape as well (Strand *et al.* 2004). Several MMPs are reported to be involved in PCD processes, through paradoxical contrasting modes of action. For example, ST3 inhibits cancer-cell apoptosis and its over-expression decreases spontaneous apoptosis in tumour xenografts (Wu *et al.* 2001). Conversely, cancer cells injected into ST3–null mice have a higher rate of spontaneous apoptosis than in wild-type hosts (Boulay *et al.* 2001). Moreover, ST3 may inhibit apoptosis by releasing IGFs, which can act as survival factors (Baserga 2000). Although in animal models ST3 decreases cancer-cell apoptosis, it increases apoptosis during tissue remodeling and development (Ishizuya-Oka *et al.* 2000). The demonstration that canine rST3 possesses unusual functional properties adds further support to the possibility of obtaining inhibitors with appropriate selectivity to target ST3 for the treatment of mammary carcinomas.

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