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Recombinant tissue inhibitor of metelloproteinase-3 from canine mammary tumor induces apoptosis *in-vitro*

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ABSTRACT

Tissue inhibitor of metelloproteinases-3 (TIMP-3) is an endogenous inhibitor of matrix metelloproteinases (MMPs). TIMP-3 is tightly bound to the extracellular matrix and it restrains the tumor growth by inhibiting matrix metelloproteinases (MMPs) as well as members of ADAM and ADMTS proteinases families by binding to their active site in a 1:1 stoichiometric ratio. The currently known TIMPs (TIMP-1, 2, 3 and 4) are very well-conserved in humans, other vertebrates, insects, and even in nematode *Caenorhabditis elegans*. The present work was carried out to produce recombinant canine (*Canis lupus familiaris*) TIMP-3 protein lacking the signal peptide in *E. coli* using expression vector pPROEXH-Tc. The TIMP-3 mature peptide encoding gene was successfully cloned and expressed in *E. coli* and the purified recombinant protein was found to be functionally active and it showed apoptotic activity on MDCK cells.

Key words: Apoptosis, Canine, *In-vitro,* Mammary tumor, Tissue inhibitor of metelloproteinases-3

Matrix metelloproteinases (MMPs) are zinc dependent endopeptidases that play important role in tumor growth, invasion and metastasis (Ala-aho *et al*. 2005). They are inhibited by specific endogenous tissue inhibitors of metelloproteinase (TIMPs) by binding to their active site in a 1:1 stoichiometric ratio (Fassina *et al*. 2000). So far, four mammalian TIMPs have been identified and they are TIMP-1, TIMP-2, TIMP-3 and TIMP-4. Among these, TIMP-3 has been identified as a unique tumor suppressor with several interesting features. It is tightly bound to the extracellular matrix, inhibits tumor cell invasion and tumor vascularisation and displays a potent proapoptotic effect on tumor cells *in vivo* (Abonen *et al*. 1998, Baker *et al*. 1999, Qi *et al*. 2003, Finan *et al*. 2006). It has also been found that the expression of TIMP-3 is silenced in various types of malignant cells (Bachman *et al*. 1999). TIMP-3 induced apoptosis and has been shown to be involved in stabilization of cell surface death receptors and activation of caspase-8 (Bond *et al*. 2002, Ahonen *et al*. 2003). Suppression of death receptor signalling by silencing death receptor and caspase-8 genes are important features in many types of malignant cells (Hopkins-Donaldson *et al*. 2003). Therefore, it is possible that the proapoptotic effect of TIMP-3 could be hampered in cancer cells. TIMP-3 also inhibits adhesion of melanoma cells to extracellular matrix (ECM) and thus may promote apoptosis via anoikis (Zhu *et al*. 2001).There are reports of reduced activities of TIMPs (1, 2 and 3) in canine mammary tumor which is significantly correlated with malignancy of tumors (Kawai *et al*. 2006). Mammary tumors are the most common tumors in un-spayed bitches. The prevalence of mammary tumors in bitches is about three times to that of women (Benjamin *et al*. 1999). Historically, about 50 percent of mammary tumors in dogs were found to be malignant (Sorenmo *et al*. 2003). There are reports of over expression of TIMP-3 in malignant mammary tumors of dogs (Klopfleisch *et al*. 2010). It suggests that this protein is specifically required for controlling extracellular matrix remodelling processes during mammary tumor progression. In the present study the expression of canine TIMP-3 protein in *E.coli* was done to study its apoptotic effect *in-vitro.*

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MATERIALS AND METHODS

Collection of tissue samples: Canine mammary tumor tissues were collected at the time of surgery carried out at the Laparoscopy Section, Veterinary Polyclinic, IVRI, Izatnagar, India, and histopathologically examined. Tumor tissues were collected under aseptic condition and immediately placed in RNA*Later* with the help of a DEPC treated scissors and forceps. Samples were stored at –20°C until further processing.

Isolation of total RNA and cDNA synthesis: Extraction of total RNA was done using total RNA Kit^{TM} following

manufacturer's protocol. The absorbance of extracted RNA was measured at 260 nm and 280 nm in spectrophotometer. The ratio of OD_{260} and OD_{280} was calculated to check purity of the extracted RNA. Complementary DNA (cDNA) was synthesised from isolated RNA sample using Oligo-dT primer and cDNA kit following the manufacturer's protocol.

Cloning, expression and purification of recombinant TIMP-3 (rTIMP-3) protein: Messenger RNA (mRNA) coding region of *Canis lupus familaris* TIMP3 gene (Accession No: JF508171.1) was used to amplify the mature sequence, using cDNA as template. The primer sequences, 5'-GCGAATTCATGCACATGCTCGCCCAGCC-3'(Forward) and 5'-CGCTCGAGTCAGGGGTCTGTGG CATTGATGAT-3' (Reverse) were chosen to amplify 567bp fragment encoding TIMP-3. The amplification was carried out in 25 µl final volume using *Pfu* DNA polymerase. After an initial denaturation at 95ºC for 5 min, 35 cycles of amplification were performed as follows: denaturation at 95ºC for 1min, annealing at 62ºC for 1min, and extension at 72ºC for 1 min followed by a final extension step of 5 min at 72°C. The PCR products were analysed in 1% agarose gel along with DNA molecular weight marker. Prokaryotic expression vector and purified PCR amplicon were digested with *Eco*RI and *Xho*1 as recommended by the manufacturer. Digested products were purified by Mini elute gel extraction kit following manufacturer's protocol. The digested insert DNA was ligated at 22°C for 2 h with the vector DNA using T4 DNA ligase. The recombinant plasmids were transformed into competent *E. coli* DH5α cells. The transformed cells were then plated on LB agar plates supplemented with ampicillin (100 µg/ml). The plates were incubated at 37°C for 12–14 hrs for the development of colonies. Colonies harbouring the recombinant plasmid were grown overnight in LB broth containing ampicillin (100 µg/ml) at 37°C with vigorous shaking. The recombinant plasmid was isolated from the culture using kit and subjected to double digestion using *Eco*RI and *Xho*1 to confirm correct orientation of the insert in the vector. Clones were sent for sequencing and sequence of TIMP-3 gene was submitted to NCBI under the accession number JX144398.1.

Transformation of pPROEXHTc-TIMP-3 in *E.coli* BL21 was done to study protein expression. Bacterial cells carrying the recombinant plasmid were grown at 37°C in LB broth with 100 µg/ml ampicillin until absorbance of the culture suspension reached 0.6 at 600 nm. Recombinant protein was expressed after four hours of induction with 1 mM IPTG at 37° C incubation with vigorous shaking. The bacterial cells were harvested by centrifugation, and the bacterial pellet was centrifuged at 12,000 rpm for 10 min at 4°C. The bacterial pellet was dissolved in urea lysis buffer pH 7.8 (8 M urea, 100 mM NaH_2PO_4, 10 mMTris-HCl) and passed through the His bound Ni-NTA column. The column was washed with excess of wash buffer pH 6 (8 M urea, 100 mM NaH_2PO_4, 10 mMTris-HCl). The bound proteins were eluted with elution buffer pH 4 (8 M urea, 100 mM NaH_2PO_4, 10 mMTris-HCl), and 1ml fractions were collected. The purity of the protein was checked by electrophoresis on SDS-PAGE using 12% gel. The refolding of the purified protein was done by dialyzing against decreasing concentrations of urea viz 6M, 4M, 3M, 2M, 1M urea and PBS to remove urea. The concentration of the recombinant protein was checked spectrophotometrically. The confirmation and purity of the recombinant TIMP-3 protein (rTIMP-3) was analysed by SDS-PAGE and western blotting by using anti-human TIMP-3 polyclonal antibodies.

Apoptosis study by flow cytometry: To study the effect of rTIMP-3 on apoptosis, MDCK cells were sub-cultured in DMEM supplemented with 100 U/ml penicillin and 100 µg/ml Streptomycin and 10% fetal calf serum and seeded in 12 well plates at the concentration of 1×10^6 cells/well. The plates were incubated at 37°C with 5% CO_2, when the cells reached a confluency of 65%, rTIMP-3 was added 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 50 nM vinblastine only, while untreated cells served as blank. The plates were finally incubated for 36 h under same conditions. After 36 h cells were harvested with trypsin versene solution and centrifuged at 3000 rpm for 2–3 min, to pellet the cells. Washing was done 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 followed by centrifugation at 3000 rpm for 3–4 min and cell pellets 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 3000 rpm for 3–4 min to remove unbound dye and pellets were resuspended in 200 µl 1× binding buffer. Finally, 10,000 cells were analyzed per sample by BD FACS Calibur instrument.

Statistical analysis: The apoptotic indices of rTIMP-3 and vinblastine treated cells were compared with untreated cells (Mock) by using the JMP 8.0 statistical package (SAS Institute 2009). The data was represented as mean ± SEM. The mean comparisons between groups were tested by Tukey's HSD test. Differences were considered significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Mammary neoplasia is one of the most common tumors in dogs and approximately half of canine mammary tumors are of malignant nature. Tumor invasion involves cellular disengagement from the local microenvironment, followed by degradation of the surrounding matrix and cellular movement (Egeblad *et al*. 2002). The ability of tumor cells to modulate the surrounding extracellular matrix (ECM) is prerequisite for induction of apoptosis. Matrix metelloproteinases (MMPs) hydrolyze the protein and proteoglycan components of the ECM. Accelerated ECM breakdown is associated with tumor invasion and metastasis (Montgomery *et al*. 1994). TIMP-3, a unique tumor suppressor, inhibits tumor cell invasion and tumor vascularization and displays a potent proapoptotic effect

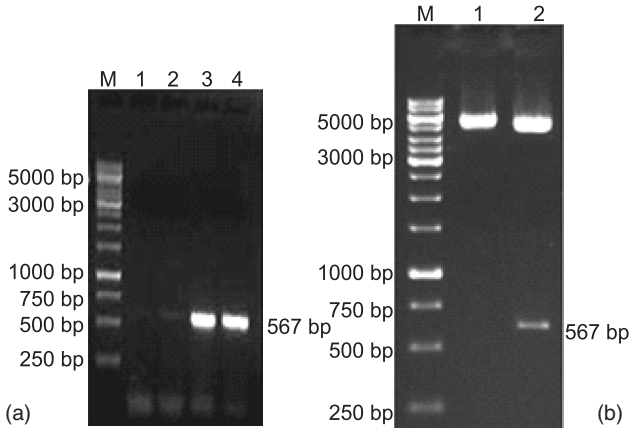

Fig.1. Amplification and cloning of truncated TIMP-3 gene (a) PCR amplification of TIMP-3. M: 1kb DNA ladder, lane 1, 2: non template control and lane 3, 4: PCR amplified TIMP-3; (b) RE digestion of pPROEX HTc.TIMP-3, M: 1kb DNA ladder, lane 1: undigested plasmid, lane 2: relese of the insert after digestion with *Eco*R1 and *Xho*1.

on tumor cells *in vivo* (Baker *et al*. 1998). Considering the significance of TIMP-3 in tumor metastasis, it was cloned and expressed in prokaryotic cells and the apoptotic activity of rTIMP-3 was checked in MDCK cells.

Putative mature form of canine TIMP-3 lacking the N-terminal signal sequence was expressed in *E. coli* BL21 using pPROEX-HTc vector. PCR amplification of cDNA yielded 567bp amplicon (Fig. 1a). The presence of insert was checked by restriction digestion of recombinant plasmid with *Eco*R1 and *Xho*1, with the release of 567bp insert (Fig. 1b). TIMP-3 mature peptide was expressed in *E. coli* BL21 as a fusion protein with 6X His tag of about 25 kDa size (Fig. 2a). The fusion protein was renatured by slow dialysis and identity of the 25 kDa fusion protein was confirmed by western blotting using commercially available rabbit anti-human TIMP-3 polyclonal antibodies (Fig. 2b).

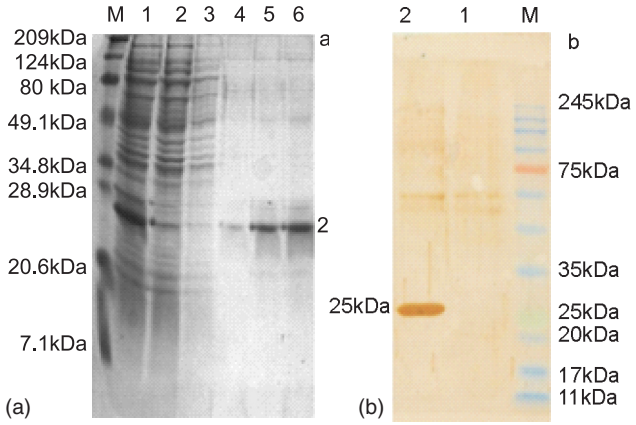

Fig.2. Purification of recombinant TIMP-3 and Western blot (a) SDS-PAGE analysis of the expressed and purified rTIMP-3 protein Lane M: Prestained broad range protein ladder (Bio-Rad) Lane 1: Cell lysate, lane 2: column flow, lane 3: wash, lane 4–6: purified rTIMP3 protein fraction collected.
(b) Western blot of rTIMP-3 using anti-human TIMP-3 polyclonal antibodies.

A well established Canine cell line MDCK cells were used to study the apoptotic effect of rTIMP-3 protein of canine origin. Apoptosis is a mode of programmed cell death with alterations in the plasma membrane triggering recognition and engulfment of such cells by phagocytes. Measurement of phosphotidyl serine (PS), an early event of apoptosis is widely followed for detection of apoptotic cells using fluorescent labelled Annexin V or Annexin V-FITC (Zafar *et al*. 2007; Logue *et al*. 2009). Annexin V detects apoptotic cells by binding with high affinity to the phosphatidyl serine (PS) exposed on the plasma membrane of apoptotic cells. In our study, rTIMP-3 from canine origin expressed in prokaryotic cells has been evaluated for its apoptotic property in MDCK cells. Induction of apoptosis by adenovirally expressed TIMP-3 in melanoma cells and inhibition in growth of human melanoma xenografts has been reported earlier (Ahonen *et al*. 2002). Adenoviral delivery of TIMP-3 have also been demonstrated to promote apoptotic cell death in SCLC cells in the absence of caspase-8 activation suggesting that TIMP-3 is a promising therapeutic anticancer protein (Kallio *et al*. 2011). As a positive control in the study, MDCK cells treated with vinblastine, a known anticancer drug has been included while the untreated cells are kept as negative control.

Distinct morphological changes in MDCK cells treated with rTIMP-3 and vinblastine were observed in phase contrast microscope as compared to untreated cells. At 50 nM concentration, more cell death was observed in vinblastine treated group as compared to rTIMP-3 treated MDCK cells. Cell death in negative control group was negligible. However, the type of cell death in all the groups whether it is apoptosis or necrosis could not be differentiated by microscopic examination. Therefore, flowcytometric analysis of cells was carried out using Annexin V-FITC and PI to determine the nature of cell death (Fig.3a,b). PI is a cell membrane impermeable DNA binding dye and generally excluded from viable cells. This dye is commonly used as a counterstain in multicolour fluorescent techniques for identifying dead cells in a population. Discrimination of apoptotic cells from necrotic cells in flowcytometry is made on the basis of affinity of both Annexin V-FITC and PI. In apoptosis, membrane changes leading to PS exposure occur rapidly followed by loss of membrane integrity in the later stage. Necrotic cells expose PS and lose membrane function simultaneously soon after cell injury (Koopman *et al*. 1994; Martin *et al*. 1995). The percent apoptotic and necrotic cells under different treatment are shown in Table 1. In negative control or untreated group, the percent cell death by apoptosis is similar to that of necrosis. In rTIMP-3 treated group, number of apoptotic cells and necrotic cells was found to be significantly higher than untreated group. When compared with positive control, vinblastine treated group, the cell death both as apoptosis and necrosis in rTIMP-3 treated group is almost half. As per the findings, when vinblastine induces 65% apoptosis, it also causes 32% necrosis. In a similar trend, rTIMP-3 causes 37% apoptosis and 13% necrosis. Higher percent of necrosis seen at FL2

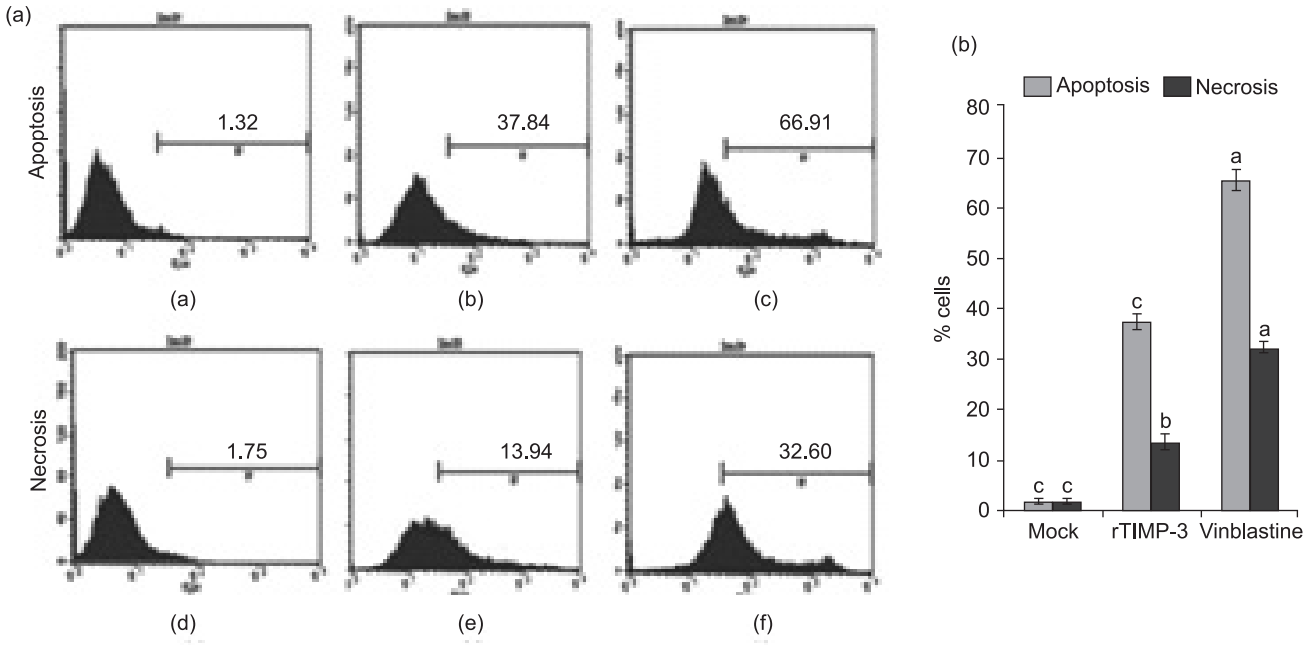

Fig. 3. Flow cytometric analysis to check apoptotic and necrotic effect of rTIMP-3 protein in MDCK cells. (a) FACS analysis by histogram plot; a, d: Mock , b, e: Cells treated with rTIMP-3, c, f: Cells treated with Vinblastine. (b) Data analysis of FACS results in MDBK cells.

Table 1. Percent apoptosis and necrosis in MDCK cells

Cell treatment	Apoptotic cells (FL1)	Necrotic cells (FL2)
Mock control (cells only)	1.5333±0.4013	1.7167±0.2892
rTIMP-3 treated	37.2267±.0391	13.4967±.1786
Vinblastine treated	65.0500±.0208	32.41±.1787

Values are mean ± standard error of 3 experiments. Values differ significantly (P< 0.05).

might be due to the fact that a number of cells undergoing apoptosis are at late stage leading to uptake of PI. It has been demonstrated that in late apoptotic cells the integrity of the plasma and nuclear membranes decreases allowing PI to pass through the membranes and exhibits red fluorescence by intercalating into nucleic acids (Rieger *et al*. 2011). At present, TIMP-3 is emerging as the most attractive anti-tumour agent. Various workers have demonstrated its promising anti-tumor effect in animal models of malignant glioma and human leukaemia xenografts in mice (Yu *et al*. 2006). To conclude with our findings, it can be stated that rTIMP-3 from canine origin has the potential for inducing apoptosis in MDCK cells and is an indicative that TIMP-3 may prove to be an anti-cancer therapeutic agent.

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