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Expression and characterization of a recombinant soluble form of bovine tumor necrosis factor receptor type I

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Abstract

A recombinant soluble bovine tumor necrosis factor receptor type I (sboTNF-RI) was expressed in the methylotrophic yeast *Pichia pastoris* and evaluated for its ability to inhibit bovine tumor necrosis factor alpha (TNF-α) cytotoxicity. A cDNA encoding the extracellular domain of bovine TNF-RI was placed under the control of the powerful and tightly regulated alcohol oxidase1 (AOX1) gene promoter of the pPICZa A vector and the resulting construct integrated into the 5′ region of the alcohol oxidase genes of GS115 and KM71 strains of *Pichia*. Soluble bovine TNF-RI was secreted into the medium following induction of the AOX1 gene promoter with methanol, and purified to greater than 95% purity by ion-exchange chromatography. In in vitro assays, the purified recombinant sboTNF-RI will block the cytolytic activity of bovine TNF-α on WEHI 164 cells clone 13 by 50% when used at a concentration of 170 μg/ml, and by nearly 90% when used at a concentration of 310 μg/ml. Results of this study suggest that recombinant sboTNF-RI may have therapeutic value as a TNF inhibitor in cattle with coliform mastitis. Published by Elsevier Science B.V.

Keywords: Tumor necrosis factor alpha; Receptor; Expression; *Pichia*; Bovine

1. Introduction

Tumor necrosis factor alpha (TNF-α) is a pleiotropic cytokine produced mainly by activated macrophages/monocytes that plays an important role in immunity, including the

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recruitment of leukocytes to injured tissues during bacterial and other microbial infections, and following stimulation with inflammatory substances (Old, 1988; Tracey and Cerami, 1994). However, when present in excessive quantities, TNF-α is known to cause tissue injury, and has been implicated in the pathology associated with several inflammatory and autoimmune diseases (Di Girolamo et al., 1997).

Two natural inhibitors of TNF-α, comprised of the soluble TNF receptors type I (TNF-RI) and II (TNF-RII) which are formed by proteolytic cleavage of the extracellular domains of the p55 and p75 TNF receptors, respectively, have been identified in urine and sera from healthy individuals, and have been shown to be elevated in some chronic diseases and following inoculation with agents that induce TNF-α release (Gatanaga et al., 1990; Van Zee et al., 1992). Genes coding for these receptors have been cloned in several species and the proteins they encode expressed in recombinant form (Dembic et al., 1990; Nophar et al., 1990). In vitro and in vivo studies using these recombinant proteins as well as receptor-Ig fusions have shown that these proteins have great potential as therapeutic agents for protecting against TNF-mediated cytotoxicity (Van Zee et al., 1996; Eliaz et al., 1996).

Bovine coliform mastitis, characterized by inflammation of the mammary gland due to intramammary infections with Gram-negative bacteria, is common in dairy cattle during the periparturient and early lactation periods, and is a major cause of lost milk production and reduced milk quality for the dairy industry (Eberhart, 1984; Smith and Hogan, 1993). Milk TNF-α levels have been shown to be greatly elevated during natural or experimental bovine coliform mastitis, while extremely high serum TNF-α concentrations have been associated with peracute disease, suggesting that TNF-α might play a major role in the pathophysiology of the disease (Sordillo and Peel, 1992; Shuster et al., 1996; Nakajima et al., 1997). To facilitate investigations of the ability of recombinant soluble bovine TNF-RI (rsboTNF-RI) to act as an inhibitor of bovine TNF-α, and to assess the therapeutic potential of rsboTNF-RI in protecting cattle with coliform mastitis against the harmful effects of TNF-α, the cDNA encoding the bovine TNF-RI was isolated and the sequence coding for the extracellular domain determined (Lee et al., 1998). In the present paper we report expression in the yeast *Pichia pastoris* of the protein encoded by the predicted bovine TNF-RI extracellular domain sequence and demonstrate that the protein has strong in vitro TNF blocking activity.

2. Materials and methods

2.1. Expression and purification of recombinant sboTNF-RI

The sequence encoding the putative extracellular domain of bovine TNF-RI was amplified by PCR from the previously cloned bovine TNF-RI cDNA (Lee et al., 1998) using the upstream primer 5′-*TCTCTCGAGAAAAGAGAGGCTGAAGCT*CTGGTCCCT-CACCCCGGG and the downstream primer 5′-GCTCTAGAGCTTATG-TAGTGCCTGGGTCCTGAGAGTCTT. The upstream primer contains the last 27 nucleotides (italicized in the primer sequence) of the sequence coding for α-factor

signal peptide from the pPICZα A vector (Invitrogen, San Diego, CA), and incorporates an *Xho* I restriction site at the 5′ end of the amplified product. The downstream primer incorporates a translation stop codon and an *Xba* I restriction site at the 3′ end of the amplified product. The amplified product was isolated using the Wizard® PCR Preps DNA Purification System kit (Promega, Madison, WI), digested with *Xho* I and *Xba* I restriction endonucleases (Gibco BRL, Grand Island, NY) and ligated into the *Xho* I and *Xba* I sites of the pPICZα A vector. Ligations were transformed into *Escherichia coli* strain Top10F′ (Invitrogen) and transformants selected according to recommended procedure (Invitrogen). Recombinant clones were analyzed for the presence of insert by restriction cleavage, and sequenced to confirm the constructs.

Sac I linearized pPICZα/sboTNF-RI plasmid DNA (5 mg/transformation) was used to transform *P. pastoris* strains GS115 and KM71 (Invitrogen) chemically according to the EasyComp™ *Pichia* Transformation Kit protocol (Invitrogen). Integration of vector DNA into the *Pichia* genome was confirmed by PCR using 5′AOX1 and 3′AOX1 primers as described in the EasySelect™ *Pichia* Expression Kit protocols (Invitrogen). The methanol utilization (Mut) phenotypes of Zeocin (Invitrogen) resistant transformants from the GS115 transformation reaction were also analyzed according to the EasySelect™ *Pichia* Expression Kit protocols.

Expression of rsboTNF-RI was performed in baffled shake flasks (Sigma, St. Louis, MO) according to recommended procedure (EasySelect™ *Pichia* Expression Kit protocols). Buffered glycerol-complex medium (BMGY) was used for the generation of biomass and buffered methanol-complex medium (BMMY) for induction of protein expression. To maintain induction, 100% methanol was added to a final concentration of 1% every 24 h. To purify rsboTNF-RI, *Pichia* culture supernatants were concentrated using Centricon Plus-80 concentrators (Millipore, Bedford, MA) and dialyzed overnight at 4°C against column equilibration buffer (10 mM Tris–HCl, 25 mM NaCl, 10 mM β-mercaptoethanol, pH 8.0). They were then loaded onto 20 ml columns of diethylaminoethyl (DEAE)-sephacel (Sigma) previously equilibrated in the same buffer. Bound proteins were eluted using a NaCl gradient (0–500 mM) in 10 mM Tris–HCl, pH 8.0. The presence of protein in each fraction was tested by monitoring absorbance at A_{280}. Fractions containing rsboTNF-RI were pooled, concentrated using a Centriprep-10 concentrator (Millipore) and stored at 70°C.

2.2. Protein analysis

Proteins were separated electrophoretically on a 15% sodium dodecyl sulfate (SDS)–polyacrylamide gel and visualized by Coomasie Blue staining or transferred onto nitrocellulose membranes, and rsboTNF-RI identified using rabbit anti-bovine whole serum adsorbed with horse and pig serum proteins (Sigma).

2.3. TNF inhibition assays

TNF inhibition assays were performed as described in the CellTiter 96™ Non-Radioactive Cell Proliferation Assay (http://www.Promega.com/tbs/tb112/tb112.html)

with slight modifications. Briefly, dilutions of rsboTNF-RI were incubated for 1 h at room temperature with equal volumes of bovine whey samples prepared as previously described (Shuster et al., 1993) from milk from quarters infused with lipopolysaccharide (LPS) from *E. coli* serotype 055:B5 (Sigma, St. Louis, MO). To serve as reference for TNF-α cytotoxicity blockade, the anti-bovine TNF-α MAb BC9 (Sileghem et al., 1992) (courtesy of Dr. Jan Naessens, International Livestock Research Institute, Nairobi, Kenya) was used at single concentration of 900 mg/ml. The samples were then assayed in triplicate for cytolytic activity on WEHI 164 mouse fibrosarcoma, clone 13, cells seeded at a concentration of 3×10^4 cells/well in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) and 50 μg gentamicin/ml (Sigma). A recombinant murine TNF-α (Endogen, Woburn, MA) was used as standard to calculate TNF-α activity in the samples. Absorbencies were read at a wavelength of 590 nm and corrected for background absorbencies using a reference wavelength of 650 nm.

3. Results

A DNA fragment encoding 181 aa of the predicted extracellular domain of bovine TNF-RI was amplified by PCR and cloned into the pPICZαA expression vector downstream of the inducible alcohol oxidase1 (AOX1) gene promoter and in frame with the sequence coding for the α-factor signal peptide. The resulting pPICZα/sboTNF-RI construct was linearized at a unique Sac I site within the 5′AOX1 region and transformed into GS115 and KM71 cells leading to integration of the vector DNA into the corresponding region of the *Pichia* genome. Integration was confirmed by PCR, and the Mut phenotype of GS115 integrants determined by growth on minimal methanol histidine plates. Several Mut^+ GS115 integrants as well as Mut^s KM71 integrants were tested for their ability to express sboTNF-RI in small-scale expression cultures. Clones, which upon induction with methanol secreted a protein of the expected size (∼21 kDa) as determined by SDS–polyacrylamide gel electrophoresis with Coomassie Blue staining (Fig. 1), were selected and used in large-scale protein expression studies.

Expressed soluble bovine TNF-RI protein was purified by ion-exchange chromatography and verified to be of bovine origin by western transfer using rabbit anti-bovine whole serum adsorbed with horse and pig serum proteins (Fig. 2). Purified rsboTNF-RI protein was evaluated for its ability to block bovine TNF-α cytotoxicity on murine WEHI 164 cells. Bovine whey samples prepared from milk from quarters infused with 25 μg LPS/quarter served as a source of bovine TNF-α. Recombinant sboTNF-RI expressed in this study showed near-linear inhibition of TNF-α cytotoxicity up to a concentration of 300 μg/ml (Fig. 3). Higher rsboTNF-RI concentrations did not result in significant changes in inhibition. From nonlinear regression (fit) of the data, 50% inhibition of TNF-α cytotoxicity was achieved at a receptor concentration of 170 μg/ml. In this study, MAb BC9, when used at a concentration of 900 μg/ml, inhibited 85% of TNF-α cytolytic activity.

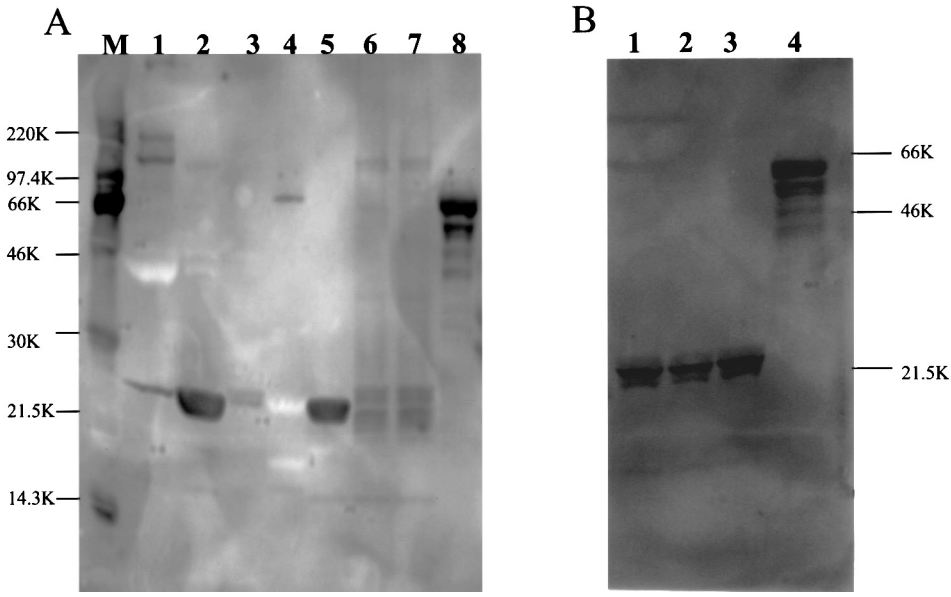

Fig. 1. SDS–PAGE (15%) analysis of the expression of rsboTNF-RI in culture supernatants of the yeast *P. pastoris* strains GS115 and KM71 after induction with methanol. Five hundred microliters of culture supernatants were concentrated to 15 µl, and the entire volume loaded onto gel. M, M.W. marker. The time points at which samples were taken for analysis are shown at the top of each lane. Arrows point at position of rsboTNF-RI (predicted size of unglycosylated protein ~20.35 kDa).

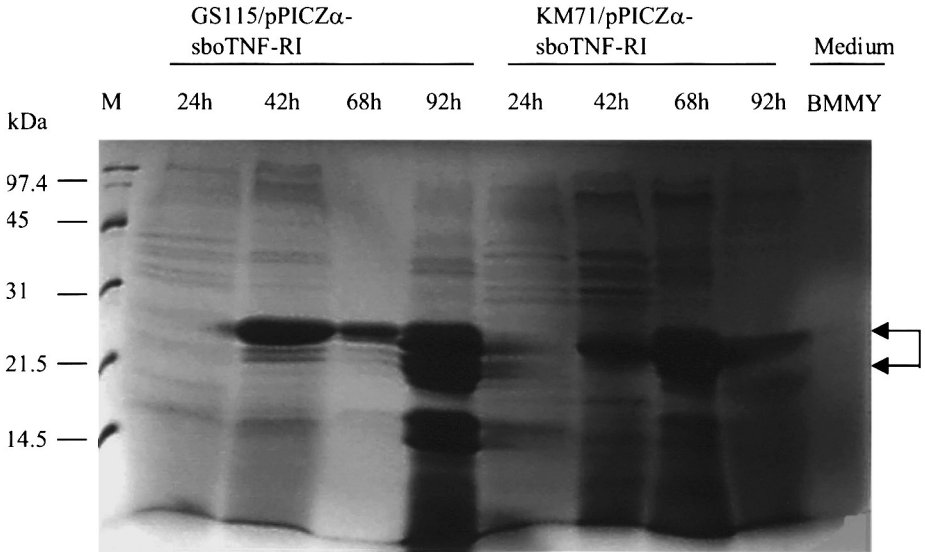

Fig. 2. Western blot analysis of sboTNF-RI in supernatants from KM71 transformants after 68 h of induction with methanol, and in ion-exchange chromatography purification fractions (B). The primary antibody used was rabbit anti-bovine whole serum. A: M, M.W. marker; 1, untransformed KM71 cells; 2–3 and 5–7, various transformants; 4, maltose binding protein fusion; 8, GS115/His$^+$ Mut^s secreted human serum albumin control. B: 1–3, purified sboTNF-RI fractions; 4, GS115/His$^+$ Mut^s secreted human serum albumin control. Arrows indicate position of sboTNF-RI.

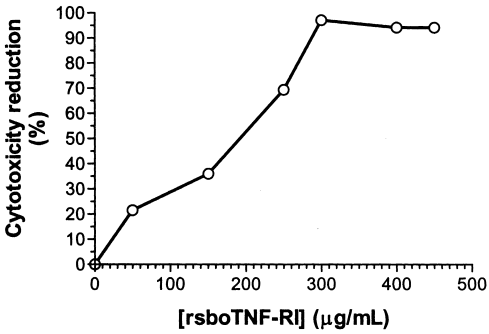

Fig. 3. Evaluation of anti-bovine TNF activity of rsboTNF-RI. Varying amounts of rsboTNF-RI were pre-incubated for 1 h at 37°C with 6.4 U bovine TNF-α/ml, the mixtures added to WEHI 164 cells and incubated for 20 h at 37°C. For control, whey samples were mixed with medium or the anti-bovine TNF-α MAb BC9. The cells were then incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and cell viability evaluated by measuring absorbance at a wavelength of 590 nm.

4. Discussion

The predicted sequence of the extracellular domain of bovine TNF-RI contains four repeating cysteine-rich motifs, each comprised of six cysteine residues, which share significant homology with the cysteine-rich domains (CRDs) of the extracellular domain of the human TNF-Rs (Loetscher et al., 1990). Although these cysteine residues have been demonstrated to play an important functional role, the high cysteine content found in these proteins has been found to create solubility problems due to improper protein folding when the receptors are expressed in prokaryotic expression hosts (Marsters et al., 1992; Hale et al., 1995; Jones et al., 1997). The *P. pastoris* heterologous gene expression system used to express sboTNF-RI in the present study has, on the other hand, been used successfully to produce, in a soluble form, large quantities of proteins such as human TNF-α that have hitherto shown insolubility when expressed in *E. coli* (Sreekrishna et al., 1989). In the present study, sboTNF-RI was expressed as a diffuse protein ranging in molecular weight from ∼20 to 25 kDa, each band probably representing a different level of glycosylation of the protein. However, despite producing variously glycosylated protein species, *P. pastoris* has been reported not to hyperglycosylate secreted proteins, which is important especially for proteins that are intended for in vivo use (Tschopp et al., 1987).

The efficacy of the expressed recombinant sboTNF-RI as an inhibitor of bovine TNF-α cytotoxicity was demonstrated by its ability to potently block the cytolytic activity of bovine whey samples, from endotoxin-infused glands, on WEHI cells even when used at relatively low concentrations. The fact that the protein inhibits bovine TNF-α nearly as well as MAb BC9 makes it a better therapeutic candidate than the MAb since anti-TNF-α monoclonal antibodies have been shown to elicit an anti-globulin response when used in vivo to block TNF-α activity (Abraham et al., 1998). Moreover, since soluble TNF-RI has previously been shown to bind both TNF-α and lymphotoxin (LT or tumor necrosis factor

β), this protein should be more valuable as an anti-TNF agent than MAbs that recognize only one form of TNF (Marsters et al., 1992).

Coliform mastitis is the major mastitis problem in well-managed dairy herds with low bulk somatic cell counts mostly due to the fact that mastitis control procedures currently in use, and which mainly entail prevention of exposure of teat canals to new infections, are ineffective against coliform bacteria infections (Smith and Hogan, 1993; Barkema et al., 1998). The problem of coliform mastitis is compounded by the fact that a significant number of intramammary infections with coliform bacteria result in a peracute disease which is accompanied by severe systemic disturbances and often death, and usually leads to premature culling of the affected animals (Eberhart, 1984; Smith and Hogan, 1993).

Several studies have reported elevated levels of TNF-α and other inflammatory cytokines in whey and serum samples from cows with acute and peracute coliform mastitis, and have suggested that TNF-α might play a pivotal role in the pathogenesis of the disease (Shuster et al., 1993, 1996; Nakajima et al., 1997). In murine and primate models of Gram-negative bacterial sepsis, TNF blockade with soluble recombinant TNF-RI, soluble TNF-RI/IgG fusions or anti-TNF antibodies was shown to protect against bacteria-induced lethality and to lessen hemodynamic disturbances as well as decrease systemic levels of other inflammatory cytokines (Hale et al., 1995; Van Zee et al., 1996). Although not evaluated in the present study, it is possible that the srboTNF-RI produced in this study could protect against the harmful effects of TNF-α during bovine coliform mastitis. Moreover, since studies in other species have shown sTNF-RI–IgG1 fusions to have higher TNF-α blocking activity than the receptor or anti-TNF-α MAbs, the development of this latter reagent will provide an even better tool for evaluation against TNF-α-induced lethality in cattle (Haak-Frendscho et al., 1994).

5. Conclusion

In summary, we have produced a recombinant soluble bovine TNF-RI that is a potent inhibitor of bovine TNF-α. The availability of this reagent in large quantities will facilitate further elucidation of the role of TNF in the pathology of various infectious diseases and its therapeutic value in preventing and treating. Work is currently underway to express a soluble bovine TNF-RI/IgG fusion protein and to evaluate its ability to block bovine TNF-α cytotocixity.

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