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Functional expression of recombinant canstatin in stably transformed *Drosophila melanogaster* S2 cells

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Abstract

We describe the expression and *in vitro* activity of recombinant canstatin from stably transformed *Drosophila melanogaster* S2 cells. Southern blot analysis indicated that transformed S2 cells contained multiple copies of the canstatin gene in the genome. Recombinant canstatin with a molecular weight of 29 kDa was secreted into the culture medium. Recombinant canstatin was purified to homogeneity using a simple one-step Ni^{2+} affinity fractionation. Purified recombinant canstatin inhibited human umbilical vein endothelial cell proliferation in a dose-dependent manner. The concentration at half-maximum inhibition (ED_{50}) for recombinant canstatin expressed in stably transformed *Drosophila* S2 cells was approximately 0.37 μg/ml. A maximum production level of 76 mg/l of recombinant canstatin was obtained in a T-flask culture of *Drosophila* S2 cells 6 days after induction with 0.5 mM $CuSO_4$.

Keywords: Recombinant canstatin; *Drosophila melanogaster* S2 cells; Expression; Purification; *In vitro* activity

Angiogenesis is the process of blood vessel formation in which new vessels develop from existing vessels. Studies have shown that tumor growth is dependent on angiogenesis [1]. Tumor angiogenesis requires both up-regulation of endogenous angiogenic stimulators, and down-regulation of endogenous angiogenic inhibitors [2]. Certain factors, including endostatin, tumstatin and canstatin are regarded as endogeneous inhibitors of angiogenesis *in vivo* [3–5].

The vascular basement membrane (VBM)[2] constitutes an important component of blood vessels and capillaries [6]. Type IV collagen is a major component of vascular basement membrane [7]. Canstatin is a C-terminal globular noncollagenous domain of the alpha 2 chain of type IV collagen that inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells [8,9]. Canstatin also inhibits tumor growth in mouse models [5] and is a potent inhibitor of angiogenesis with a distinct antitumor activity [5,9].

An efficient expression system needs to be developed to produce the large quantities of soluble canstatin necessary for early clinical trials. Recently, canstatin expression was reported in *Escherichia coli* and 293 embryonic kidney cells [5]. However, expression of recombinant canstatin by stably transformed insect cells has not yet been examined. We describe stable expression of the cDNA for human canstatin in *Drosophila melanogaster* S2 cells and purification of recombinant canstatin using metal chelate affinity fractionation. We also describe the *in vitro* activity of recombinant canstatin derived from stably transformed S2 cells.

Materials and methods

Cell lines, plasmids, and enzymes

Drosophila melanogaster Schneider 2 (S2) cells were grown at 27 °C in a T-25 culture flask (Nunc, Denmark)

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[1] They equally contributed to this paper.
[2] *Abbreviations used:* VBM , vascular basement membrane; S2, Schneider 2; IMS, insect medium supplement; HUVEC, human umbilical vein endothelial cells; CID, collision-induced dissociation; bFGF, basic fibroblast growth factor.

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in Shields and Sang M3 Insect Medium (Sigma, USA) containing 10% (v/v) IMS (Insect Medium Supplement; Sigma). The 3.6 kb pMT/BiP/V5-His plasmid (Invitrogen, USA) contained a metallothionein promoter, BiP signal sequence, V5 epitope tag, and polyhistidine region. The selection plasmid pCoHygro (Invitrogen), which contained the bacterial hygromycin B phosphotransferase gene under the control of the constitutive *Drosophila* Copia 5′-LTR promoter, was used for stable transformation. *E. coli* DH5α was used as the primary host for constructing and propagating plasmids. *E. coli* cells were routinely grown with agitation at 37 °C in LB medium [1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.3)] containing 50 μg/ml of ampicillin. We used DNA restriction enzymes from Promega (USA) and Takara (Japan) according to the manufacturers' instructions.

Construction of expression plasmids

Human canstatin cDNA was amplified from total RNA extracted from human umbilical vein endothelial (HUVEC) cells (Clonetics, USA) by reverse transcription-PCR using oligonucleotide primers. The sense primer was 5′-AGATCTGTCAGCATCGGCTACCTCCTGGTGA AGCACAG-3′ and the antisense primer was 5′-CT CGAGCAGGTTCTTCATGCACACCTGGCAGCGGC TG-3′. The amplified canstatin sequence was then inserted into the pGEM-T vector (Promega) to yield pGEM-T-Can, and the sequence was verified by DNA sequencing. PCR was carried out using a Thermal Cycler (PE Biosystems, USA) with PCR Mix (Takara) in a volume of 50 μl. pMT/BiP/Can-V5-His was constructed by inserting the *Bgl*II–*Xho*I fragment of pGEM-T-Can between the *Bgl*II and *Xho*I sites of pMT/BiP/V5-His (Fig. 1). The proper orientation and reading frame of the insertions in pMT/BiP/Can-V5-His were confirmed by both restriction enzyme mapping and DNA sequencing.

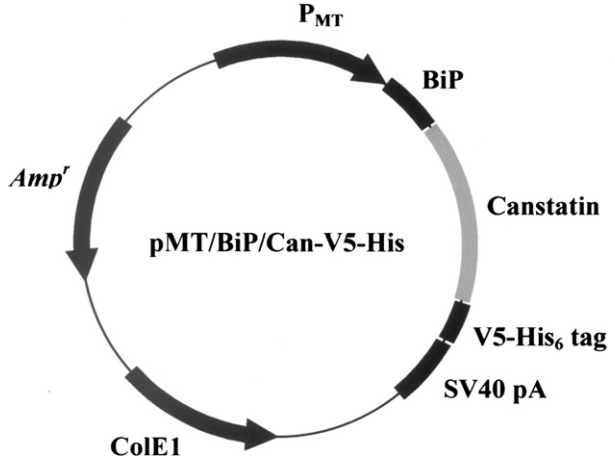

Fig. 1. Schematic representation of the expression plasmid pMT/BiP/Can-V5-His.

Stable transformation

Exponentially growing S2 cells were co-transfected with the pMT/BiP/Can-V5-His and pCoHygro plasmids (10:1) using the lipofectamine method. For preparation of the transfection medium, plasmid DNA and the lipofectamine reagent (Invitrogen) were diluted separately in IMS-free M3 medium, and then mixed at a 1:5 ratio. The transfection medium was incubated at room temperature for 45 min, then transferred into 6-well plates pre-seeded 2 h earlier with S2 cells in IMS-free M3 medium. After a 24 h incubation, the medium was changed to remove the lipofectamine, and the cells were incubated for 5 additional days without drug selection in M3 medium containing 10% IMS. The cells were then centrifuged and resuspended in selective M3 medium containing 10% IMS and 300 μg/ml hygromycin B (Duchefa Biochemie BV, Netherlands). The selective medium was replaced every 5 days and stably transformed polyclonal cell populations were isolated after 4 weeks of selection with hygromycin B. Hygromycin B was maintained continuously in the media after the selection procedure.

Cell culture and analysis of gene expression

Stably transformed S2 cells expressing recombinant canstatin were grown at 27 °C in Nunc (T-25) flasks in 5 ml of M3 medium containing 10% IMS and 300 μg/ml hygromycin B. Stably transformed S2 cells were cultured for 8 days in Nunc flasks to analyze cell growth and canstatin expression. Canstatin expression was induced by addition of 0.5 mM $CuSO_4$ after the start of the run.

The cultures were centrifuged at 3000 rpm for 5 min and the supernatants were used to identify extracellular recombinant proteins. The cell fraction was incubated with rocking for 30 min in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml of PMSF, 1 μg/ml of aprotinin, and 1% Triton X-100], and subjected to three freeze-thaw cycles of freezing at −70 °C followed by incubation at 37 °C in a water bath. The cell extracts were centrifuged at 14,000 rpm for 15 min to remove cell debris, and the supernatant was used to identify intracellular recombinant proteins. A mixture of the intracellular and extracellular fractions was used to analyze total protein production, unless specified otherwise.

Southern blot analysis

Genomic DNA was prepared from both non-transfected and stably transformed cells using a Wizard genomic DNA purification kit (Promega), and 0.2 μg aliquots were digested with *Bgl*II and *Xho*I. A probe was prepared from a 684-bp *Bgl*II and *Xho*I fragment of pMT/BiP/Can-V5-His, including part of the coding region for human canstatin. The digests and equivalents were resolved on 0.8% agarose gel and transferred to Hybond-N membranes following the manufacturer's instructions (Amersham-Pharmacia Bio-