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Research paper

Expression and purification of canine granulocyte colony-stimulating factor (cG-CSF)

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ABSTRACT

Canine granulocyte colony-stimulating factor (cG-CSF) with modification of cysteine at position 17 to serine was expressed in *Brevibacillus choshinensis* HPD31. cG-CSF secreted into the culture medium was purified by ammonium sulfate precipitation and consecutive column chromatography, using butyl sepharose and DEAE sepharose. Biological activity of the recombinant cG-CSF was 8.0×10^6 U/mg protein, as determined by its stimulatory effect on NFS-60 cell proliferation. Purified cG-CSF was subcutaneously administered once a day for two successive days to dogs (1, 5, 25, or 125 μg). Neutrophil count increased the following day in all dogs except those administered the lowest dose (1 μg). No severe side effects were observed in dogs after administration of cG-CSF.

1. Introduction

Neutropenia is a major side effect in dogs that undergo cancer chemotherapy, causing infection, ultimately leading to morbidity and mortality. Therefore, high doses of anti-cancer drugs cannot be used for chemotherapy in small animal practice. Treatment with nontoxic inducers of neutrophils would clearly be beneficial for accelerating recovery or preventing chemotherapy-induced myelosuppression. Granulocyte colony-stimulating factor (G-CSF) is a lineage-specific growth factor that primarily influences production and differentiation of neutrophils, with its use sometimes resulting in shortened periods of neutropenia in patients receiving intensive chemotherapy (Baker et al., 2000; Dexter et al., 1990; Dunn and Goa, 2000; Johnston and Crawford, 1998). In veterinary medicine, recombinant human G-CSF (hG-CSF) approved for human use has been used in animals. Unfortunately however, administration of hG-CSF in dogs and cats for a few weeks results in development of neutralizing antibodies not only to heterologous hG-CSF but also to homologous G-CSF, leading to severe neutropenia (Hammond et al., 1991; Lothrop et al., 1988; Phillips et al., 2005). Thus safe, non-immunogenic G-CSF therapeutics are needed for small animal practice.

Bacillus brevis, reclassified as *Brevibacillus choshinensis* (Shida et al., 1996), has been established as an expression system with the advantage that large amounts of recombinant proteins are secreted directly into culture medium with little or no detectable proteases or endotoxins (Udaka et al., 1989; Tanaka et al., 2001). Secreted proteins are usually correctly folded and hence biologically active, thus refolding of recombinant proteins is not required thereafter and the purification scheme can be simplified.

In this study, expression of cG-CSF in *B. choshinensis* HPD31-M3 strain was attempted to obtain large amounts of cG-CSF for practical use. After modification of the cG-CSF protein, cG-CSF retaining high biological activity was

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Abbreviations: WBC, white blood cell; G-CSF, granulocyte colony-stimulating factor; DEAE, diethylaminoethyl; PVDF, polyvinyl difluoride.

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successfully produced. Purified cG-CSF was further evaluated both *in vitro* and *in vivo*.

2. Materials and methods

2.1. The expression vector (pNY326) for recombinant cG-CSF

pNY326 was constructed as follows. P5 promoter and signal sequence of HWP was amplified from the cell wall protein gene of *B. choshinensis* from pNU210 (Udaka and Yamagata, 1993) using primers P5M and P5RV (Table 1). HS gene for termination of RNA transcription was amplified from *B. choshinensis* genomic DNA using primers HSM and HSRV (Table 1). These fragments were cloned into pGEM-7zf, and a fragment containing both genes was prepared then inserted into pUB110 (McKenzie et al., 1986), with the bleomycin gene deleted. The signal peptide sequence of MWP was modified from the original sequence to R2L6 (Sagiya et al., 1994) for up-regulation of protein expression and secretion. The resultant plasmid was designated as pNY326.

The DNA fragment encoding mature cG-CSF (GenBank accession No. I36329) was synthesized by two-step assembly PCR (Withers-Martinez et al., 1999) with four synthetic DNAs of 153 nucleotides containing 30 bp overlaps with each other. PCR was conducted as follows: denaturation at 95 °C for 2 min, followed by 25 cycles at 95 °C for 45 s, 55 °C for 1 min and 72 °C for 2 min, and a final elongation step at 72 °C for 5 min. The full-length molecule was selectively amplified from 5 μL of step PCR mixture using DNA5, DNA6 primers (Table 1) and cloned into pGEM®-T vector (Promega, Madison, USA). After the nucleotide sequence was confirmed, the ORF was amplified using CaGCSF2-5′, CaGCSF3 + H6-3′ primers (Table 1) and cloned into pET3d vector (pET-cGCHis). The cG-CSF ORF fragment was amplified using cGCSFSM and cGCSFRV primers (Table 1) using pET-cGCHis as template. Amplified DNA was digested with NcoI and BamHI, then cloned into expression vector pNY326. The resultant plasmid was designated pNY326-cG-CSF. pNY326-cG-CSF was introduced into *B. choshinensis* HPD31-M3 by electroporation (Udaka and Yamagata, 1993). *B. choshinensis* HPD31 carrying pNY326-cG-CSF (named Bb/cG) was cultivated in 20 L of T2Nm medium (1% polypeptone, 0.2% yeast extract, 0.5% meat extract, 1% glucose, and 50 μg/mL neomycin, pH 7.0) for 65 h. The broth was centrifuged, then filtered with a 0.22 μm membrane to remove bacteria.

Table 1
Nucleotide sequence of primers.

DNA5	5′-gCCCCCCTgggCCCTACCgg-3′
DNA6	5′-gggTTTggCAAAgTggCgCA-3′
CaGCSF2-5′	5′-ggCCATggCCCCCCTgggCCCT-3′
caGCSF3 + H6-3′	5′-CCggATCCTCAgTgATggTgATgATgATggCCggg-TTTggCAAAgTggCgCA-3′
cGCSFSM	5′-AACCATggCTTTCgCTgCCCCCCTgggCCCTACC-ggCCCCCTgCCCCAgAgCTTCCTgCTCAAgAgCCT-3′
cGCSFRV	5′-CgggATCCTCAgggTTTggCAAAgTggC-3′
P5M	5′-TTTgCCgTCAggggAATATACTAg-3′
P5RV	5′-gAgTCTAgATCgACggATC-3′
HSM	5′-AAgCTTAATTCCTATCCAggAT-3′
HSRV	5′-ATgCATTCgCAgTTCCCAT-3′

2.2. Purification of cG-CSF from culture medium

Ammonium sulfate powder was added to the medium with continuous stirring until 40% saturation. The precipitate was collected by centrifugation, then dissolved in 100 mL phosphate buffer (pH 8.0). Ammonium sulfate was added to 20% saturation, then the precipitate pelleted by centrifugation. Supernatant was applied to a Hi-prep butyl sepharose FF column (20 mL, prepacked for FPLC, Pharmacia, Piscataway, NJ, USA). The column was washed with 10 mM phosphate buffer (pH 8.0) followed by elution with a linear gradient from 20% to 0% ammonium sulfate in 10 mM phosphate buffer (pH 8.0), 3.3% sorbitol. Fractions containing cG-CSF were pooled and applied to a Hi-trap DEAE sepharose FF column (5 mL, prepacked for FPLC, Pharmacia, Piscataway, NJ, USA). The column was washed with 10 mM phosphate buffer (pH 8.0), 3.3% sorbitol followed by elution with 50 mM NaCl, 10 mM phosphate buffer (pH 8.0), 3.3% sorbitol. Endotoxins were removed from the eluent containing cG-CSF with an ultrafiltration 100 kDa membrane (Millipore TFF system™). Final samples contained a maximum of 0.3 EU/mL of endotoxin.

2.3. Western blotting

In brief, canine G-CSF was separated by SDS-12.5% PAGE, then electrotransferred from gels onto PVDF membranes (Millipore Corp., MA, USA). Membranes were blocked with blocking buffer (3% skim milk in PBS) for 1 h, then incubated for 1 h with rabbit anti-feline G-CSF antiserum (Yamamoto et al., 2001). After washing with PBS, membranes were incubated with horseradish peroxidase-conjugated protein A (Dako Japan, Co. Ltd., Kyoto, Japan) for 1 h. Bands were visualized by addition of 0.5 mg/mL diaminobenzidine and 0.05% H_2O_2 in PBS.

2.4. In vitro assay of G-CSF activity

Mouse myeloid leukemia cell line NFS-60 (Weinstein et al., 1986; kindly provided by Professor K. Nagata, Osaka University, Japan) was cultured in DMEM containing 10% fetal bovine serum and 1 ng/mL recombinant murine IL-3 (Pepro Tech EC, Ltd., NJ, USA). G-CSF activity was measured by a proliferation assay using NFS-60 cells (Yamamoto et al., 2001; Shirafuji et al., 1989) in a 96-well microtiter plate. Cell growth was monitored using a Cell Counting kit-8™ (Dojindo, Kumamoto, Japan) after staining with WST-8 tetrazolium salt. G-CSF titer (U/mL) is defined as the reciprocal of the dilution value that gave 50% of cell growth.

Feline G-CSF (fG-CSF) in culture supernatant from Sf21 infected by recombinant baculovirus was established as a laboratory standard (Yamamoto et al., 2001).

2.5. In vivo assay of G-CSF activity

Eight healthy Beagle dogs (8 months, 10 kg) were purchased from Nosan Co., Ltd. (Yokohama, Japan). Four groups (two dogs per group) were administered subcutaneously with either 1, 5, 25, or 125 μg purified cG-CSF. Blood samples were collected from each dog before,