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Molecular Cloning, Expression and Purification of G-CSF Isoform D, an Alternative Splice Variant of Human G-CSF

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ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is the major regulator of hemopoiesis and granulopoiesis. However, overexpression of G-CSF has been implicated in several important processes in tumor biology such as tumor growth, angiogenesis, and metastasis. Four different mRNA isoforms resulting from alternative splicing have been reported for G-CSF (transcript variants 1, 2, 3 and 4). The mRNAs and protein products of splice variants 1 and 2 have been isolated for the first time, from tumor cell lines. In the present study for the first time we isolated the G-CSF transcript variant 4 encoding G-CSF isoform D from a highly malignant tumor cell line (Mehr80) with overexpression of G-CSF.

Both the full-length G-CSF isoform B and G-CSF isoform D were cloned from Mehr80 cell line, overexpressed in *Escherichia coli* as N-terminal glutathione-S-transferase fusion proteins in the form of inclusion bodies and affinity purified by the batch method using glutathione-Sepharose 4B resin.

Both fusion proteins were successfully cloned and expressed. Folded recombinant proteins were solubilized from inclusion bodies using sarkosyl, Triton X-114 and CHAPS and purified. The purity of G-CSF isoforms was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and they were clearly detected in western blot analysis using anti-G-CSF polyclonal antibody.

The G-CSF plays various roles in physiological and pathological conditions, however to date, the differential function of G-CSF isoforms remains unknown. Considering the fact that G-CSF isoform D was isolated from a highly malignant tumor cell line with overexpression of G-CSF, the role of this splice variant in tumorigenesis requires further investigation.

Keywords: Alternative splicing; Glutathione S-transferase; Granulocyte colony stimulating factor; Leukocytosis; Malignant tumors; Recombinant fusion protein

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INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is the major regulator of hemopoiesis and granulopoiesis produced by a variety of cell types. In addition to neutrophil proliferation and survival, G-CSF plays a key role in the mobilization of neutrophil and hematopoietic progenitor cells from the bone marrow. G-CSF was rapidly integrated into clinical medicine for treatment of chemotherapy-associated neutropenia and hematopoietic progenitor cells transplantation.[1,2]

G-CSF gene contains five exons. Four different mRNA isoforms resulting from alternative splicing have been reported for G-CSF. While transcript variants 1 and 2 include all five exons, they utilize two donor splice site at the 5' terminus of intron 2 arranged in tandem, 9bp apart. Transcript variant 1 encodes the mature 177-amino acid G-CSF isoform A and transcript variant 2 encodes the major biologically active G-CSF isoform B consisting of 174 amino acids.[3] The G-CSF isoform B is the most studied and the major isoform produced in prokaryotic and eukaryotic expression systems for therapeutic proposes. Two other G-CSF isoforms, 141-amino acid isoform C and 138-amino acid isoform D, are respectively encoded by transcript variants 3 and 4 which both skip exon 3 (108bp) and use two donor sites at 5′ splice site of the second intron (Figure 1).

Besides the crucial role of G-CSF in innate immunity, interestingly, overexpression of G-CSF has been implicated in several important processes in tumor biology.[4-7] Paraneoplastic leukemoid reaction characterized by extreme leukocytosis has been described in a variety of malignancies and in numerous cases; it has been linked to high G-CSF production by the tumor.[8] In normal cells G-CSF production is highly regulated however, aberrant production of significant amounts of G-CSF as a consequence of the abnormal processing of G-CSF mRNA has been reported in various malignant cell lines.[9]

G-CSF overexpression in many solid tumors is considered as a poor prognostic marker however, the mechanisms remained unclear.[10] Upregulation and mutations of G-CSF receptor (G-CSFR) have also been implicated in a variety of hematopoietic and non-hematopoietic malignancies indicating the pivotal role of G-CSF/G-CSFR signaling axis in the pathogenesis of these malignancies.[9,11-13]

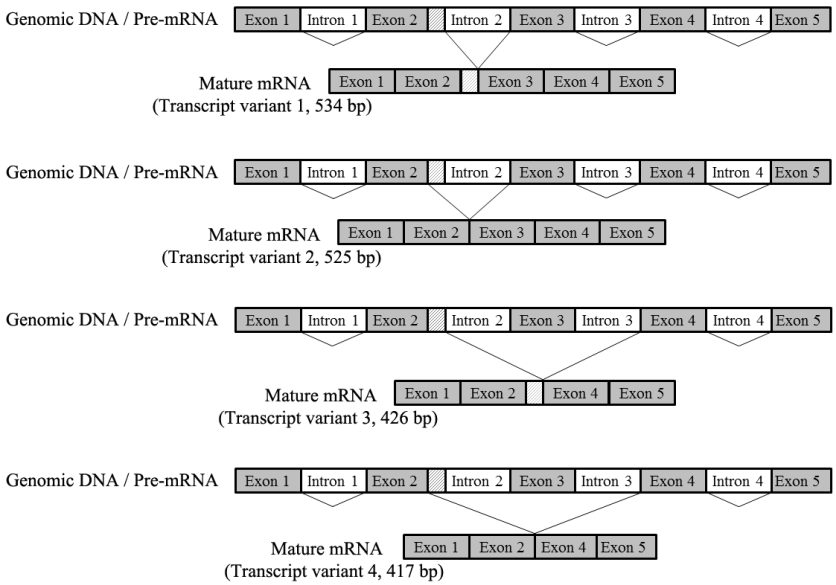

Figure 1. Schematic representation of alternative splicing events in the human granulocyte colony-stimulating factor (G-CSF) gene. Alternative splicing of G-CSF pre-mRNA, containing five exons, generates four different mRNA isoforms (transcript variants 1, 2, 3, and 4). In the transcript variants 1 and 2 all five exons are included, while two splice sites are utilized at the 5' terminus of the second intron. Transcript variants 3 and 4 are produced by alternative exclusion of exon 3 and use of alternative 5′ splice site of intron 2. Transcript variants 2 and 4 were cloned in the present study. Solid lines indicate splicing options

Two splice variants of G-CSF have been purified for the first time from tumor cell lines, isoforms A from squamous cell carcinoma cell line CHU-2,[14] and isoform B from the human bladder carcinoma cell line 5637.[15] Later, Nagata et al cloned the cDNAs encoding both G-CSF isoforms A and B from the CHU-2 tumor cell line.[3] The molecular mechanisms determining alternative choices of exons and splice sites remained unknown. We also detected transcript variant 4 mRNA for the first time in a G-CSF producing tumor cell line.[16] Changes in alternative splicing pattern under pathological conditions including cancers have been reported in many genes. Regarding the dual role of G-CSF in tumorigenesis, there is a possibility that just as many other transcript variants in various types of cancers, changes in the balance of G-CSF transcript isoforms occur during oncogenesis. Identification of tumor specific splice isoforms even those with an unknown function may have diagnostic and prognostic value and also can be considered as a promising therapeutic target in different malignancies.[17]

In this study, we reported the isolation of a shorter splice variant of G-CSF, transcript variant 4. Both isoforms B and D were cloned from Mehr80 cell line and overexpressed in *Escherichia coli* (*E. coli*). To the best of our knowledge, this is the first report regarding the cloning, heterologous expression, and purification of G-CSF isoform D.

MATERIALS AND METHODS

Cell Culture and RNA Extraction

This project was approved by the local ethics committee of Shiraz University of Medical Sciences, Shiraz, Iran (N. IR.SUMS.REC.1393.S6991).

Mehr-80 cell line is an adherent lung large cell carcinoma established in Shiraz Institute for Cancer Research.[16] The cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin (Biosera, France), and 100 µg/mL streptomycin (Biosera, France) in humidified 5% CO_2 at 37°C. Total RNA was extracted from Mehr-80 cell line using High Pure RNA Isolation Kit (Roche Diagnostics, Germany) according to the manufacturer's instruction and cDNA was generated from about 1µg of the total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Lithuania).

Isolation and Cloning of Two Splice Variants of Human G-CSF

To isolate the mature cDNA of full-length human G-CSF (GeneBank Accession No. NM_172219.2), one pair of primers, forward primer 5′-ATGACCCCCTGGGCCCTG -3′ and the reverse primer 5′- TCAGGGCTGGGCAAGGTGG-3′ was employed. The polymerase chain reaction (PCR) was carried out under the following conditions: initial denaturation temperature of 94ºC for 3 min, then 30 cycles of 30 s at 94ºC, 30s at 62 ºC, 1 min at 72ºC with a final extension for 10 min at 72ºC using a T100 Thermal cycler (Bio-Rad, USA). Although PCR conditions were optimized, unexpectedly, we found two bonds with lengths of about 400bp and 500bp on agarose gel. We decided to clone both PCR products. The amplified products were cloned into *EcoRI* and *BamHI* restriction sites of the pUC19 vector (Thermo Fisher, USA) and sequenced on both strands to determine their identities. The resulting plasmids were named pUC19-G-CSF/B and pUC19-G-CSF/D.

Construction of Expression Plasmids

The DNA fragments coding for full-length G-CSF isoform B (174 amino acids) and alternatively spliced shorter isoform of G-CSF (isoform D, 138 amino acids) were amplified by PCR from pUC19-G-CSF/B and pUC19-G-CSF/D. PCR amplifications were performed by using forward primer containing *XhoI* restriction site and reverse primer containing *EcoRI* restriction site. The recombinant genes encoding mature proteins were then inserted into the expression vector pGEX-4T-1 (GE Healthcare, USA), at the same restriction sites downstream of the glutathione-S-transferase (GST)-tag sequence. The sequences of the resulting pGEX-GST-G-CSF/B and pGEX-GST-G-CSF/D plasmids were confirmed by DNA sequencing.

Expression and Purification of Human G-CSF Isoforms in *E. coli*

The expression and purification of recombinant G-CSF isoforms were performed according to a method previously described with modifications.[18] *E. coli* BL21 (DE3) (Novagen, USA) competent cells were transformed with recombinant pGEX-GST-G-CSF/B and pGEX-GST-G-CSF/D plasmids and were grown at 37 ºC in 10 mL of LB medium containing 100 µg/mL ampicillin. When absorbance at 600 nm reached 1, the culture broths were diluted 1:20 in 200 mL fresh LB

medium containing 50 µg/mL ampicillin, 1% glucose, 440 mM sorbitol and 2.5 mM betaine and incubated at 37ºC until the cell density reached an OD_{600} of 0.5. To induce protein expression, isopropyl-b-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.25 mM after cooling the cultures to 27 ºC. Cultures were incubated overnight at 27ºC with 180 rpm shaking to allow for protein expression. The cells were harvested at 4ºC and analyzed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The bacterial pellets were suspended in cold lysis buffer containing 50 mM Tris-HCl pH 8.0, 1M NaCl, 20 mM 2-mercaptoethanol (2-ME) and 1 mg/mL lysozyme and incubated for 45 min at 4°C. Sarkosyl was added to a final concentration of 1%, and the mixture was incubated at room temperature for 2 hours. The cell lysate was centrifuged at 15000g for 20 min at 4°C to discard the cell debris. Just before purification, the solution was incubated with 2% Triton X-114 and 20 mM CHAPS at 4°C for 30 min. The sample was then transferred to a 37°C water bath, incubated for 10 min, and centrifuged at 20,000 g for 10 min at 25°C. The supernatant containing soluble GST-fusion protein was mixed gently by the batch method with 2 mL (bed volume) of glutathione-Sepharose 4B at 4°C for 2 h. The nonspecifically bound proteins and the remaining detergents were removed by washing with binding buffer (50 mM Tris-HCl pH 8.0, 500mM NaCl, 20 mM 2-ME) 4 times. The target protein was eluted in about 10 mL of 10 mM glutathione in 50 mM Tris-HCl at pH 8.0. The eluted fraction was dialyzed against phosphate buffered saline (PBS) pH 7.3 for overnight at 4°C. The protein concentration was determined by the Bradford method with bovine serum albumin as the reference standard. To check the presence of detergents, the absorbance of wash and elution fractions was measured at 240 nm.

SDS–PAGE and Western blot Analysis

The induced cultures, non-induced cultures, and eluted fractions were subjected to 12.5% SDS-PAGE, and protein bands were visualized by Coomassie Brilliant Blue staining. For western blotting, samples were separated by 12.5% SDS-PAGE gels and transferred to polyvinyl difluoride (PVDF) membrane. The membrane was incubated with rabbit anti-human G-CSF (1:2000 dilution, polyclonal antibody, Abcam, USA) and then incubated with Horseradish peroxidase-conjugated anti-rabbit IgG (1:6000 dilution, Abcam, USA) as secondary antibody. The peroxidase activity was detected by enhanced chemiluminescence (Bio-Rad, USA).

RESULTS

Cloning and Sequence Analysis of Two Alternatively Spliced Variant of Human G-CSF

There are four alternative splicing variants of the G-CSF gene. Using specific primers designed for full-length human G-CSF, PCR was expected to yield a 525bp product; however, two bands of 417bp and 525bp were identified (Figure 2). Sequence analysis and Basic Local Alignment Search Tool (BLAST) results revealed that the larger 525bp amplicon corresponded to the full-length transcript of G-CSF consisting of five exons (transcript variant 2), whereas deletion of exon 3 (108bp) was detected in the shorter amplicon of 417bp (transcript variant 4, Accession No. NM_001178147). We deposited this sequence in GenBank under Accession No. HM132049. While transcript variant 2 encodes for full-length mature G-CSF isoform B with 174 amino acids, the transcript variant 4 encodes a mature protein with 138 amino acids (G-CSF isoform D).

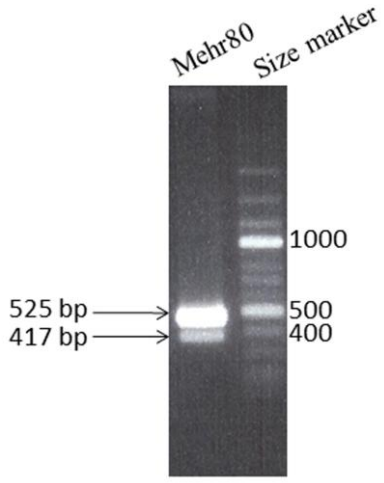

Figure 2. The reverse transcriptase polymerase chain reaction (PCR) analysis of mRNA prepared from Mehr80 cell line using specific primers of granulocyte colony-stimulating factor (G-CSF) transcript variant 2. Band of 525 bp represents the full length G-CSF transcript variant 2, while that of 417 bp represents the G-CSF transcript variant 4.

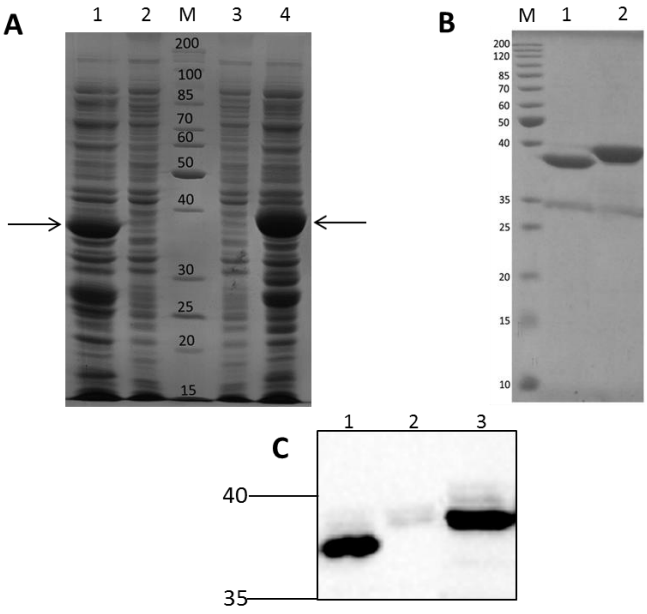

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of granulocyte colony-stimulating factor (G-CSF) isoforms B and D. (A) The 12.5% SDS–PAGE analysis of expression of recombinant glutathione-S-transferase (GST) fusion proteins in the total cell lysates prepared by boiling in SDS loading buffer. Lane 1, recombinant GST-G-CSF isoform D; Lane 2, non-induced (without IPTG) control of GST-G-CSF isoform D; Lane 3, non-induced (without IPTG) control of GST-G-CSF isoform B; Lane 4, recombinant GST-G-CSF isoform B. (B) The 12.5% SDS–PAGE analysis of purification of recombinant GST-G-CSF isoform D (lane 1) and GST-G-CSF isoform B (lane 2). (C) Two fusion proteins were examined by western blot after a 12.5% reducing SDS-PAGE performance. Lane 1, recombinant GST-G-CSF isoform D; Lane 2, non-induced (without IPTG) control; Lane 3, recombinant GST-G-CSF isoform B.

Expression and Purification of Recombinant Human G-CSF Isoforms

To express human G-CSF isoforms in *E. coli* BL21 (DE3), corresponding genes without any codon sequences optimization were cloned into the pGEX-4T-1 vector. Using this expression plasmid, the G-CSF isoform B and isoform D were produced as the N-terminal GST fusion proteins. To improve conformation of the recombinant proteins, low expression temperature (27ºC) and reduced IPTG concentration (0.25mM) were used.[19] The LB media were supplemented with sucrose and betaine which function as osmolytes and help to proper folding of the recombinant protein.[18] As revealed by SDS–PAGE analysis, the expression level of both isoforms was high (Figure 3A). However most of the proteins were produced in the form of inclusion bodies. The GST-G-CSF isoforms were purified in a single-step procedure. Using 2% Triton X-114 and 20mM CHAPS, we successfully purified GST-G-CSF isoform B (protein concentration 250 µg/mL) and isoform D (protein concentration 180 µg/mL). The purity of G-CSF isoforms was confirmed by SDS-PAGE (Figure 3B). The purified GST-G-CSF proteins were clearly detected in western blot analysis using anti-G-CSF polyclonal antibody (Figure 3C). By washing with the binding buffer, the three detergents were removed from the purified proteins.

DISCUSSION

In the present study for the first time we isolated, cloned and overexpressed the G-CSF transcript variant 4 from a G-CSF producing cell line. The corresponding coding region of both full length G-CSF isoforms B and exon-skipped isoform D without signal peptide was amplified, inserted into the pGEX-4T-1 vector and successfully overexpressed in *E. coli* as GST-fusion proteins.

The *E. coli* expression system has the advantages of

high productivity, being relatively simple, rapid and inexpensive. However, the lack of eukaryotic post-translational modification machinery and formation of insoluble recombinant proteins in form of inclusion bodies are the major challenges of the prokaryote expression system.[20] In human G-CSF, there is a glycosylation site at Thr-133 which protects the molecule from aggregation and proteolytic degradation. However it has been shown that glycosylation as a post-translational modification does not affect the G-CSF biological activity. Filgrastim, a non-glycosylated form of recombinant G-CSF produced in *E. coli* is clinically available for treatment of neutropenia and stem cell mobilization for decades.[2]

We tried to express recombinant G-CSF isoform D in *E. coli* without any tag or with N-terminal and C-terminal His-tag however, it was not expressed at detectable levels. One probable reason is the high GC contents of N-terminal region of native G-CSF which could not be transcribed in *E. coli*.[21] Codon bias can also affect the expression of heterologous genes. We performed codon optimization of the G-CSF isoform D gene in order to reduce N- terminal GC content and to eliminate rare codons. But the yield of the recombinant protein was low, and we could not obtain enough amount of protein for purification. It has been shown that while codon optimization approach can improve heterologous protein production, some other factors such as mRNA secondary structure particularly, secondary structures at the 5′-UTR have an important role in efficient protein production. With using a well-expressed N-terminal protein fusion such as GST, G-CSF isoform D was successfully expressed.[22] We used GST as a fusion expression partner for two main reasons: First, GST as one of the most common fusion tags greatly enhances the expression of difficult-to-express recombinant proteins by allowing the efficient initiation of translation.[23] High levels expression of the GST-fusion proteins in *E. coli* may result in the formation of inclusion bodies, as we observed in our experiment. The production of inclusion bodies can, however, be considered an advantage as inclusion bodies allow the overproduction of the highly pure recombinant proteins.[24] Second, GST is a very commonly used affinity tag allowing for one-step purification of many proteins.[25] Although in our experiment the recombinant GST-G-CSF isoforms were produced in the form of inclusion bodies, by using Sarkosyl, proteins were solubilized from inclusion bodies. In the presence of sarkosyl, GST- fusion proteins are not properly folded and could not be affinity-purified. Therefore, it is necessary to remove sarkosyl from the lysate before purification. As described by Tao et al Triton X-100 and CHAPS at a specific ratio, remove sarkosyl molecules from the solution and enhance protein folding and purification. By multiple batch mode washes of the resin, the concentration of detergents decreased significantly.[18,26]

Full-length G-CSF belongs to the four-helix-bundle class of cytokines with long connecting loops between helices A and B and between helices C and D. There is also a short helix (helix E) between helices A and B.[27] G-CSF isoform D lacks exon 3 (108 bp) encoding the amino acid residues of N-terminal region of D helix and AB loop region of the full length G-CSF. Homology modeling and docking study confirmed the stable binding of isoform D to G-CSFR through several residues on the A and C helices which are conserved in isoform D.[28] However, it should be investigated if deletion of an entire exon and its corresponding amino acids in the resulted protein can affect the G-CSF signaling and biological activity.

Exon skipping similar to that of G-CSF isoform D has been reported in other members of four-helix bundle cytokines including IL-6, IL-4 and IL-2. Bihl et al reported an alternatively spliced IL-6 mRNA lacking exon 4 which encodes a smaller isoform named IL-6δ4. Both the full-length and IL-6δ4 has been detected in the healthy lung samples. The IL-6δ4 isoform binds to IL-6 receptor α (IL-6Rα); however it lacks signaling activity. Their results showed that the short isoform may have a regulatory role in IL-6 signaling.[29] A specific inhibitory function has also been described for a splice isoform of IL-4 (IL-4δ2) generated through alternative splicing of exon 2. Luzina et al demonstrated the secretion of IL-4δ2 by activated T lymphocytes from patients with asthma, but not from healthy controls.[30] Recombinant IL-4δ2 is able to inhibit the full-length IL-4-induced effects in T lymphocytes.[30,31] While IL-4 is known as a Th2 cytokine, an independent role for IL-4δ2 as a Th1 cytokine has been proposed by some studies.[31,32] A similar mechanism of regulation has been shown for two splice variants of IL-2, IL-2δ2 and IL-2δ3, lacking exon 2 and exon 3, respectively. Both splice variant function as competitive antagonists of IL-2 inhibiting T cell proliferation.[33]

We isolated G-CSF splice variant 4 from Mehr80

cell line derived from peritoneal effusion of a female patient with lung large cell neuroendocrine carcinoma, a very rare tumor with poor prognosis.[16] While endogenous G-CSF has been known as the main regulator of granulopoiesis,[1] aberrantly expressed, tumor-derived G-CSF represents a key inflammatory component that facilitates tumor growth, angiogenesis and metastasis. The majority of G-CSF producing malignant tumors are encountered in patients with advanced-stage cancer, most of which are associated with extremely poor prognosis. Overexpression of G-CSF has been shown to be a strong inducer of proangiogenic factor Bv8 and Cd11b+Gr1+ myeloid-derived suppressor cells (MDSCs) which are important mediators of tumor angiogenesis, tumor metastasis and immune suppression. G-CSF produced by the tumor or stromal cells has a major role in tumor resistance to anti-VEGF therapy.[4-7] Regarding the pro- and anti-tumor role of G-CSF and the fact that changes in alternative splicing are associated with various pathological conditions; it is worthy to identify the occurrence and changes in the expression level of G-CSF isoforms at mRNA and protein level. Of interest, alternative splicing products of G-CSF gene and their corresponding protein isoforms has been deposited into the NCBI's Reference Sequence (RefSeq) database; however we could not find any study assessing the expression of G-CSF splice variants at mRNA and protein level. One possible reason is that the expression levels of G-CSF splice isoforms may be limited compared with that of biologically active full-length G-CSF at mRNA and protein level, not allowing for detection of these transcript variants with commonly used methods. Indeed, unavailability of anti-G-CSF antibodies reacting selectively with a specific G-CSF protein isoform without cross-reactivity to other isoforms makes it difficult to discriminate between G-CSF isoforms.

In the present study we could successfully clone, express and purify two alternatively spliced variant of G-CSF. Production of recombinant G-CSF isoform D provides the possibility of functional assessment of this isoform in future studies. Considering the fact that G-CSF isoform D was isolated from a highly malignant tumor cell line with overexpression of G-CSF, the role of this splice variant in tumorigenesis requires further investigation. It is also needed to be clarified whether isoform D has any regulatory effect on the function of full length G-CSF or its effects are independent of the effects of full-length G-CSF.

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