Scientific paper

Human Granulocyte Colony Stimulating Factor (G-CSF) Produced in the Filamentous Fungus *Aspergillus niger*

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Abstract

For the first time, a fungal production system is described for expression and secretion of the medically important human protein G-CSF, in *Aspergillus niger*. A reliable strategy was chosen with in-frame fusion of G-CSF behind a KEX2 cleavage site downstream of the coding region of the highly secreted homologous glucoamylase. This provided secretion levels of 5–10 mg/l culture medium of correctly processed G-CSF, although the majority of the protein (>90%) was biologically inactive. Following denaturation/ concentration and chromatographic separation/ renaturation, the G-CSF proliferation activity increased considerably, and analytical immobilised metal affinity chromatography confirmed the monomeric and correctly folded protein. These data suggest that this human secretory protein secreted into the medium of *A. niger* was not correctly folded, and that it escaped the endoplasmic reticulum folding control systems. This is compared to the folding of G-CSF produced in bacteria and yeast.

Keywords: Human granulocyte colony stimulating factor; filamentous fungus *Aspergillus niger*; secretion; protein folding; renaturation during chromatographic separation

1. Introduction

Human granulocyte colony stimulating factor (G-CSF) is a cytokine that has an important role in the myeloid lineage of haematopoiesis.[1] Due to its demonstrated efficacy against different forms of neutropenia and chemotherapy-induced leukopenia, as well as its suitability for allogeneic and autologous bone-marrow transplantation and peripheral blood stem-cell mobilisation, G-CSF has wide pharmaceutical applicability. There are two forms of natural G-CSF that have been isolated from tumour cell lines: the short form, which is composed of 174 amino acids and is more active; and the long form, which has three more amino acids.[2,3] The G-CSF molecule contains two disulphide bonds that stabilise the fully functional tertiary structure, and one free cysteine residue that is partially hidden in the interior, and partially exposed.[4] The active form of G-CSF is a monomer with a four α-helix crystal structure. The 18.8 kDa protein part is O-glycosylated, and has a short carbohydrate chain at Thr133, which represents ~4% of the molecular weight.

Due to its pharmacological potential, human G-CSF has already been expressed in bacteria (*Escherichia coli*), yeast, a mammalian cell line (Chinese hamster ovary [CHO] cells), and plant cell cultures.[3,5–7] For clinical use, G-CSF is available in two forms: a non-glycosylated, *E. coli* derived form (with Met on the N-terminus) and a glycosylated, CHO cell derived form that is indistinguishable from its natural counterpart.[8] Glycosylation of G-CSF does not appear to be essential for its biological activity, but it has been shown to be beneficial for its *in vitro* stability; the O-linked glycosyl group protects the G-CSF protein against molecular aggregation and protease degradation in human serum.[9–11] The non-glycosylated G-CSF produced in *E. coli* is a relatively hydrophobic protein, with an isoelectric point of ~6.1, and with high stability at low pH (pH 3–4).[12]

To date, to the best of our knowledge, there has been no successful expression of G-CSF in filamentous fungi. This system is convenient for commercial production of heterologous proteins, especially because of the natural

ability of the filamentous fungi to secrete high amounts of a variety of proteins. Indeed, many products from *Aspergilli* have obtained the 'Generally Regarded as Safe' status, like for large-scale production of enzymes.[13] Although today most therapeutic proteins are produced in mammalian cell cultures or prokaryotic systems, depending on the nature and molecular mass of the protein, fungi still hold promise as an alternative expression system. Here, we report on the secretion and isolation of correctly processed, folded and biologically active G-CSF in the filamentous fungus *Aspergillus niger*.

2. Experimental

2. 1. Construction of Expression Plasmids

The engineered cDNA gene for mature G-CSF (174 amino acids plus Met at the N-terminus, modified to incorporate useful restriction sites)[2,3] was amplified from the BBG 13 vector (R&D Systems). Oligonucleotide primers were designed to add extensions at both ends of the DNA fragment. The *NarI* restriction site (underlined here) was followed by a hexapeptide linker for the KEX2 processing site (bold here), and this was added to the 5'-end of G-CSF (5'-gtcatggcgccg**aacgtgatttccaagcga**atgaccccctgggccct), with the *HindIII* restriction site underlined here) at the 3'-end (5'-gacctaagcttgaattcggatccttatcagg). The PCR amplification conditions were: 94 °C for 30 s; 55 °C for 30 s; 72 °C for 60 s; 30 cycles. The PCR-derived fragment was ligated in-frame to the truncated *A. niger glaA* GII sequence (514 amino acids) in the pAN56-2M shuttle vector (*Nar*I and *Hind*III digestion), a derivative of the plasmid pAN56-2 (GenBank: Z32690).[14,15] The dominant selection marker *amdS* gene from *Aspergillus nidulans* was inserted into the *Not*I site of the expression vector (pAN56-2MGKA).[16] In addition, the control expression vector pAN56-2MGA was constructed, in which *glaA* GII and G-CSF were fused without a cleavage site in between them, with a different 5'-end primer used for PCR amplification (5'-gacatggcgccgatgaccccctgggccct).

2. 2. Transformation, Clone Selection and Production

2. 2. 1. Transformation

A protease-deficient strain of *A. niger*, AB1.13, was used as the production host.[17] Transformation of *A. niger* protoplasts was performed according to the chemical method.[18]

2. 2. 2. Clone Selection

Control GA transformants (pAN56-2MGA vector) and KA transformants (pAN56-2MGKA vector) were selected through two rounds of a single-spore purification process, by growing them for 6 days at 30 °C on plates that contained acetamide, and later on acrylamide (for more gene copies); however, the exact copy number of the heterologous gene was not determined.

2. 2. 3. Production

Spores for the inoculums were collected in sterile physiological solution from potato dextrose agar plates (Difco) and added to the media at a final concentration of 3×10^5 conidia/ml. Routine cultures were performed using maltodextrine (MD) minimal medium supplemented with 25 g/l maltodextrine (Sigma Dextrine Corn type I; D-2006). The MD medium containing the surfactant 0.2% (v/v) Tween 80 was also tested. Uridine (2 mM) was added to all of the media to supplement the auxotrophic mutation of the host strain. Cultures of 100 ml were grown in 500-ml flasks that were shaken (180 rpm/min) at 30 °C. The production time point of 40 h was determined experimentally according to the growth/production curve.

2. 3. Purification of Secreted Recombinant G-CSF

2. 3. 1. Sample Preparation

After 40 h, the MD medium fermentation broth was filtered using 388 filter paper (Sartorius) and a 0.45 μm HV Durapore (PVDF) filter. Then 6 M guanidinium/ HCl was added to the filtrate (final concentration, 4.3 M guanidinium/ HCl), which was concentrated approximately 10-fold using an Amicon stirred-cell device and YM-10 membrane, at 4 °C. After 18 h, the concentrate that contained 4.3 M guanidinium/ HCl was subjected to chromatographic separation.

2. 3. 2. Chromatographic Purification of Secreted Recombinant G-CSF

Chromatographic separation was carried out on an HPLC AKTA Purifier system (GE Healthcare). The UNICORN 5.11 software (GE Healthcare) was used for instrumentation control and data acquisition. The chromatographic runs were carried out at room temperature.

2. 3. 3. Size-exclusion Chromatography of the Native and Denatured Filtrate

Size-exclusion chromatography (SEC) of the native and denatured filtrate was performed using a 16/60 Superdex 75 PG SEC column (GE Healthcare) equilibrated with 2–3 column volumes of 5 mM Na phosphate, 50 mM NaCl, pH 7.0. The separation was carried out at a flow rate of 1 ml/min, with detection by UV absorbance at 280 nm; 1 mL fractions were collected continuously from the outlet stream. The fractions corresponding to the G-CSF

monomer were pooled and the pH was adjusted to 4.0. The pool was concentrated using Amicon Ultracel 10k regenerated cellulose, and prior to immobilised metal affinity chromatography (IMAC), the pH of the (native) sample was adjusted to 7.01 using 1 M Na_2HPO_4.

2. 3. 4. Immobilised Metal Affinity Chromatography

The IMAC was performed on Zn-IDA Chelating Sepharose Fast Flow chromatographic medium (GE Healthcare), equilibrated in the loading buffer (buffer A: 25 mM Na phosphate, 150 mM NaCI, pH 7.0). The column was washed with 1.5 column volumes of buffer A, and a decreasing linear pH gradient was applied over 5 column volumes, as 0% to 100% buffer B (25 mM Na phosphate, 150 mM NaCI, pH 4.0) in buffer A.

2. 4. Analysis of Recombinant G-CSF

2. 4. 1. Protein Concentration Determination

Protein concentrations were determined according to Bradford,[19] using an Agilent 8453 diode array spectrophotometer and an *E. coli* derived G-CSF standard calibration curve.

2. 4. 2. SDS-PAGE Gel Staining and Western Blotting

SimplyBlue SafeStain (Invitrogen) or Silver Stain sensitive kits (Bio-Rad) were used for staining SDS-PAGE gels, according to the manufacturer instructions. Western blotting was performed on nitrocellulose membranes using primary goat anti-G-CSF polyclonal antibodies (R&D Systems), secondary rabbit anti-goat IgG antibodies (Sigma), and 4-cloronaphthol development reagent (Sigma).

2. 4. 3. Carbohydrate Detection

The non-glycosylated G-CSF produced by *E. coli* was used as the standard control at known concentrations. Carbohydrate detection was performed using Immun-Blot kits for glycoprotein detection (Bio-Rad), according to the manufacturer instructions.

2. 4. 4. N-terminal Determination

Protein amino terminal sequences were analysed using an Applied Biosystems Model 492 pulsed-field liquid sequenator.

2. 4. 5. *In Vitro* Biological Activity

The biological activity of G-CSF was measured as *in vitro* proliferation activity on the NFS-60 murine myeloblastic cell line, as reported previously.[20]

3. Results

3. 1. Expression of Recombinant G-CSF

The G-CSF gene was introduced into the pAN56-2M shuttle vector that contained an inducible and highly efficient glucoamylase (GLA) promoter, a signal sequence, and the sequence of the first 514 amino acids of the *A. niger* GLA (*glaA GII*) gene, which codes for the GLA catalytic domain and a highly O-glycosylated linker.[14,15] The hexapeptide Asn-Val-Ile-Ser-Lys-Arg that contains a KEX2 processing site (underlined here) was introduced between the GLA gene and the G-CSF cDNA, to allow *in-vivo* cleavage by the furin-type protease KexB, and the control construct for the GLA–G-CSF fusion protein without any cleavage site was also prepared. Transcription termination and polyadenylation signals were provided by the *A. nidulans trpC* 3'-untranslated region.[14,15]

The AB1.13 protease-deficient strain of *A. niger* was used as a production host, and out of 50 clones, 32 well-growing and sporulating transformants were selected in two rounds of spore purification (KA transformants). Twenty-four clones were chosen for the expression studies, and were screened for the best production levels. Different levels of secretion of G-CSF into the growth media in most cases was confirmed by Western blotting. Out of 37 control (GA) transformants, only 12 well-growing and sporulating transformants were selected, and for two of them, the GLA–G-CSF fusion protein was detected in the growth media by Western blotting. The extracellular concentration of the KA transformant GLA–G-CSF was estimated at ~5 mg/l MD culture after 40 h of fermentation (Figure 1), while the yield of the control GA GLA–G-CSF fusion protein appeared to be considerably higher.[21] At longer times of fermentation, no further increase in the KA transformant GLA–G-CSF was observed due to increased amounts of a smaller band that appeared, which was considered as a degradation product (as shown by Western blotting). The N-terminal amino-acid sequence analysis of the processed GLA–G-CSF fusion protein confirmed the Met-Thr-Pro-Gly-Xxx-Pro sequence,

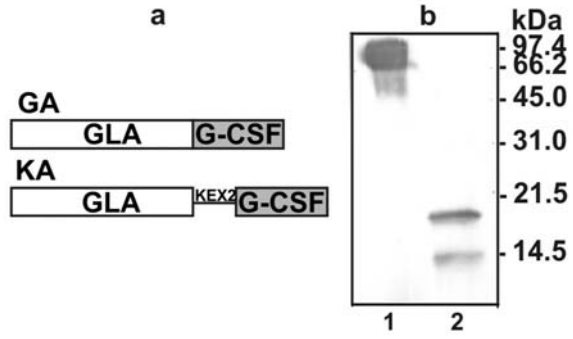

Figure 1. Expression of the recombinant G-CSF in *A. niger.*
a. Schematic representation of the gene constructs used.
b. Representative Western blot of the filtrates (18 µl): **1.** GA; **2.** KA; Right: molecular weight standards (kDa) (BioRad).

which corresponds to the designed N-terminus of G-CSF. The quantity of G-CSF secreted into the MD medium supplemented with 0.2% (v/v) Tween 80 was almost double that for the MD medium without 0.2% (v/v) Tween 80 (data not shown).

3. 2. Carbohydrate Detection

In the media tested, the secreted G-CSF had a similar size to the non-glycosylated G-CSF from *E. coli* (Figure 2a, lane 2), although strict inspection of some of the SDS-PAGE gels suggested that the G-CSF from the *A. niger* filtrate has a slightly higher molecular weight (Figure 2a, lane 3). To detect the carbohydrate constituents, Immun-blot kits for glycoprotein detection were used that were optimised for positive detection of carbohydrate structures in glycoprotein molecules (Bio-Rad). These kits use a specific carbohydrate oxidation reaction to label the carbohydrate part of the molecule with biotin, and the subsequent detection was based on streptavidin alkaline phosphatase. Although the loading of the G-CSF from the filtrate corresponded to 3-fold to 5-fold the detection level declared by the manufacturer, and although the ovalbumin used as the positive control gave the expected result, no glycosylation of G-CSF could be confirmed (Figure 2b**,** lane 1). The ovalbumin exists as mono-N-glycosylated protein at Asn292, the glycosylation site is occupied by at least 45 different glycan structures and the two of the most abundant forms contain 7 or 9 sugar glycan structures.[22] For the highly glycosylated band of high molecular weight protein observed in this sample, it is reasonable to believe that it belongs to the native GLA secreted by the fungus, in addition to the GLA GII form as consequence of the cloning strategy.

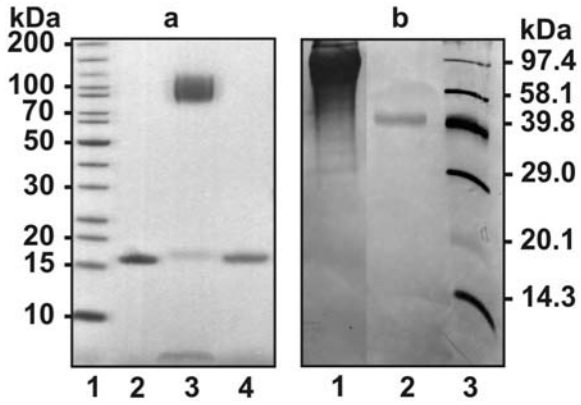

Figure 2. Analysis of the purified G-CSF and glycoprotein detection.
a. Representative analysis of the purified G-CSF on a Simply Blue stained SDS-PAGE gel: **1.** PAGE Ruler molecular weight standards (kDa) (Fermentas); **2.** *E. coli* derived G-CSF standard (1 µg); **3.** KA filtrate; **4.** Renaturated and purified G-CSF from *A. niger* (0.65 µg).
b. Representative Western blot for glycoprotein detection: **1.** KA filtrate (18 µl); **2.** Ovalbumin as the glycoprotein control (0.5 µg); **3.** Biotinylated molecular weight standards (kDa) (BioRad).

3. 3. Purification of G-CSF

Purification and isolation of the G-CSF in the culture filtrate of *A. niger* was performed using SEC (Figure 3a). When the concentrated culture filtrate without any other pre-treatments (designated as the native sample) was applied to the Superdex 75 PG SEC column, all of the G-CSF present was eluted in the same broad peak together with GLA (Figure 3b). This peak corresponded to a considerably higher molecular mass than expected for the G-CSF monomer, a clear sign that the G-CSF was in an aggregated multimeric form. To avoid protein aggregation and filter clogging, prior to the SEC, the culture filtrate was concentrated in the presence of a strong denaturing agent: 6 M guanidinium/ HCl (final concentration, 4.3 M guanidinium/

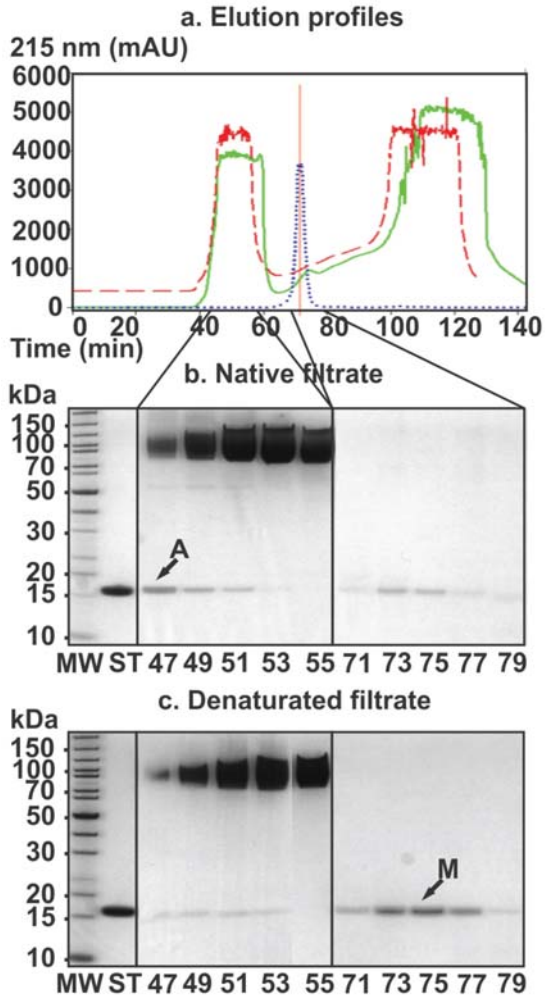

Figure 3. Size exclusion chromatography of the native and denaturated filtrates.
a. Representative elution profiles for the native filtrate (red dashed line), the denaturated filtrate (green full line) and the G-CSF standard from *E. coli* (blue dotted line).
b, c. Representative silver stained SDS-PAGE gels of the selected elution fractions from 47 to 79 (elution time (min)), for the native filtrate (b) and the denaturated filtrate (c). **A**, aggregate; **M**, monomer; left: PAGE Ruler molecular weight standards (kDa) (Fermentas).

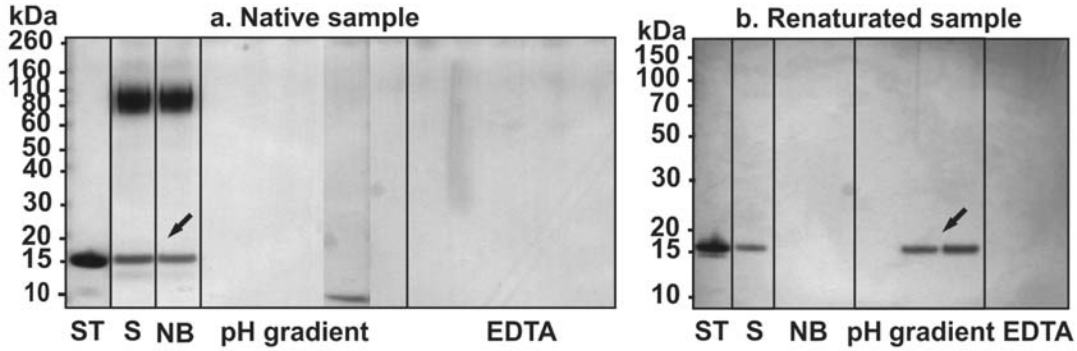

Figure 4. IMAC chromatography of the native filtrate and renatured samples. Representative silver-stained SDS-PAGE gels.
a. Native filtrate. **b.** Renatured sample. **ST**, G-CSF standard from *E. coli*; **S**, starting sample; **NB**, non-bound fraction; **pH gradient**, elution fraction using the pH gradient. Arrows, elution of G-CSF from native or renatured samples seen in completely different fractions. left: PAGE Ruler molecular weight standards (kDa) (Fermentas).

HCl); here the situation was very different. The completely denatured protein was separated and spontaneously renatured during the chromatographic separation under native conditions, using the same SEC column and buffer system as described above. When applying this denaturation–renaturation procedure, the majority of the G-CSF was eluted in the monomeric form (Figure 3c), which corresponded to the *E. coli* derived G-CSF standard.

Immobilised metal ion affinity chromatography on Zn-charged chelating Sepharose was used to determine the correctness of the G-CSF protein folding. When the native sample was applied to IMAC, practically no G-CSF bound to the column, and it eluted together with GLA without being retained (Figure 4a). In contrast, the majority of the G-CSF from the small SEC peak obtained during the separation of the initially denatured sample indeed bound to the IMAC column and eluted during a decreasing pH gradient, at the position corresponding to the *E. coli* derived G-CSF standard (Figure 4b).

3. 4. Biological Activity of G-CSF

The *in vitro* biological activity measurements of the G-CSF were based on stimulation of cell proliferation of the murine myeloid leukaemia NFS-60 cell line. Serial dilutions of the World Health Organisation reference standard (NIBSC, 88/502) and the test samples were added into the culture medium. After 48 h incubation, the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added, which was reduced by the mitochondrial dehydrogenase from the live cells into insoluble formazan crystals, and solubilized afterwards. The absorbance values measured at 570 nm represented the natural logarithm of the cell concentration. The test sample activity was determined by comparison of the dilutions of the reference standard and the test sample at which 50% of the maximal stimulation of proliferation was achieved. The proliferation activity of the native G-CSF in the *A. niger* filtrate reached approximately 7.5% of the reference standard activity, while the proliferation activity of the G-CSF obtained after the denaturation/ renaturation step from the SEC column was increased to approximately 65%.

4. Discussion

4. 1. Expression Strategy

The filamentous fungus *A. niger* is well known for its exceptionally high capacity to secrete various homologous proteins. Filamentous fungi also share some further properties that give them advantages over to other expression systems, such as: correct processing and folding of protein products, efficient disulphide bridge formation, and less over-glycosylation than observed in yeast. *Aspergillus niger* transformants are usually stable, because the incoming DNA readily integrates into the genome. Although several heterologous proteins have been successfully expressed in filamentous fungi (see[23] for an overview, and references therein), the secretion yields of therapeutic proteins still rarely reach the quantities obtained in yeast systems such as *Pichia pastoris* and *S. cerevisiae*; in these yeast systems, recombinant protein accumulation can reach up to a few hundred milligrams, or even grams, per litre.[24–27] To date, it has not been possible to create a universally perfect folding environment for recombinant proteins, and thus one of the main bottlenecks in their production is the inability of these 'foreign' polypeptides to reach their native conformation in the host cells.[28]

To overcome these problems, we chose a well-known and reliable strategy for the production of G-CSF, which involved fusion of the target gene downstream of the coding region of a highly secreted homologous gene. However, this approach is not always successful, or indeed, completely understood, and it appears that post-translational modifications and processing issues can result in major limitations to high-level production and secretion of foreign proteins.[28] Enhanced yields of heterolo-

gous proteins from *A. niger* have been achieved by fusion of the target gene downstream of either full-length *glaA* or truncated *glaA*.[29,30] Glucoamylase is a secretory glycoprotein, with bulky N-linked carbohydrate groups attached to Asn171 and Asn395, but not to Asn182, which directs the correct folding of GLA, and facilitates its secretion and enhances its thermo-stability.[31] In our case, the G-CSF was expressed behind the GLA promoter, which was fused in-frame, with the truncated *glaA* and KEX2 cleavage site in-between. We believe that in the case of this GLA–G-CSF expression, the much larger and correctly folded fusion partner, GLA, with its bulky N-glycosylation pattern, was responsible for 'tricking' the quality control system of the endoplasmic reticulum and for protecting the not perfectly folded G-CSF from proteolytic degradation, thus allowing the whole fusion protein to move to the Golgi apparatus. N-glycosylation by itself has already been used as a tool to enhance protein secretion: e.g., secretion of cutinase, a lipase, and llama V_{HH} antibody fragments by *S. cerevisiae* and *P. pastoris* improved following the introduction of an N-glycosylation site.[32] An elegant solution was found to increase the production of chymosin in *A. niger*, by introducing N-glycosylation sites outside of the native molecule, on a linker that separated prochymosin from its carrier molecule.[33]

For the artificial KEX2 linker, we used a Ser/Asn-Val-Ile-Ser-Lys-Arg sequence, which is similar to the cleavage site of the native GLA pro-region. Endoproteolytic cleavage of the G-CSF from the GLA–G-CSF fusion protein in *A. niger* proved to be accurate, and mature G-CSF was in the culture filtrate. The processing of fusion proteins carrying the Lys-Arg sequence requires KexB (although alternative endoproteases can also cleave protein fusion at sites adjacent to Lys-Arg).[34] *Aspergillus niger* KexB has an identical late-Golgi retention signal as endoprotease Kex2 from *S. cerevisiae*, which is considered to be a late-Golgi marker.[35,36] Taking all of this into account, it is very likely that the G-CSF was processed from the fusion protein in the Golgi apparatus, which means it passed the quality control system in the endoplasmic reticulum. There is only a limited chance that the G-CSF entered the filtrate in any other way than secretion, e.g., via broken hyphae, because there was no increase in the content of other intracellular proteins in the filtrate on the silver-stained gels.

4. 2. Production of Recombinant G-CSF

The protease-deficient host strain, *A. niger* AB1.13, was chosen as the host for this production of G-CSF. The selection for the transformants was performed in MD medium, where the best clones produced ~5 mg G-CSF per litre culture filtrate after 40 h of growth. The better production conditions proved to be MD medium supplemented with the non-ionic surfactant Tween 80 (0.2%; v/v). This was as expected, as Tween 80 has already been reported to improve the production of recombinant G-CSF in the yeast *S. cerevisiae* and *P. pastoris*, primarily by preventing multimerisation of the secreted G-CSF.[37–41] In contrast to the almost 100-fold increase in secretion efficiency achieved in *P. pastoris*, however, in *A. niger*, the addition of Tween 80 only moderately improved the G-CSF levels (i.e., a 2-fold increase). It is known that addition of Tween 80 and other surfactants in general negatively affects the stability of hydrophobic proteins[42] (and our unpublished data), as hydrophobic proteins such as G-CSF bind to surface active compounds.[43] These compounds offer some protection against agitation-induced protein aggregation, as shown in the case of human growth hormone and albutropin, and they are still widely used as protein pharmaceutical formulation excipients.[44,45] Thus, Tween 80 is also included in the formulation of the commercially available G-CSF produced in *E. coli* (Neupogen, Amgen, USA), although at a very low concentration (0.004%). In the next generation G-CSF drug known as Neulasta (Amgen, USA), the high micelle molecular weight Tween 80 has been replaced by the less hydrophobic Tween 20.

The yield of recombinant G-CSF expressed in *A. niger* was not very high, although it was also not negligible in terms of the secretion of a human protein in *Aspergillus* spp.. The secretion level is comparable to levels achieved in the yeast *P. pastoris*.[46,39,41] However, bearing in mind the exceptionally high secretion capacity of filamentous fungi, much higher levels of G-CSF were expected, with yields of secreted proteins from non-fungal sources usually lower in comparison to proteins of fungal origin.[23,30] So far, we have only used shaking flask fermentations with various growth media to raise the expression/ secretion levels (data not shown). Instead, yields might be improved through alterations to some of the fermentation parameters, such as temperature, agitation/ mixing, pH, and osmolality, or by switching to more controlled bioreactor conditions. Improvements might also be achieved with a producing strain that has no auxotrophic mutation (i.e., no uridine supplement needed).

4. 3. Isolation of Recombinant G-CSF

As Tween 80 is known to interfere with the purification process and its addition did not considerably improve the expression/ secretion levels, MD medium without this surfactant was chosen as the source for this isolation of recombinant G-CSF. Nevertheless, problems were encountered with the preparation of the sample, as it adsorbed to nitrocellulose filters during dialysis and filtering, which indicated that the recombinant protein is very hydrophobic. G-CSF has almost 50% hydrophobic amino-acid residues in its primary structure, and is thus a hydrophobic protein in itself, and its extremely high tendency for aggregation, the inefficiency of the surfactant addition to the medium, and the low biological activity were all suspec-

ted to be the consequence of the incorrect folding of the protein. The efficient renaturation we achieved after complete denaturation certainly supports this assumption. Namely, when the G-CSF–containing culture filtrate was concentrated in the presence of the denaturating agent guanidinium hydrochloride, renaturation and separation were successfully performed in the same step using a SEC column and non-denaturing buffers.

4. 4. Comparison of Recombinant G-CSF Produced in Bacteria and Yeast

We have previously observed similar problems relating to G-CSF for its expression in the methylotrophic yeast *P. pastoris*, particularly in terms of its presence in a soluble, yet highly aggregated, form that interferes with its purification.[46] With this yeast, the G-CSF soluble aggregates were kept together by their hydrophobic interactions, and to a lesser extent, by their intermolecular disulphide bonds. However, with this *A. niger* expression, there are practically no disulphide bonded aggregates. In *P. pastoris*, the most efficient way to obtain the monomeric, biologically active G-CSF protein was through its complete denaturation by guanidinium hydrochloride or urea, and then its subsequent renaturation using dilution or chromatographic separation.[46] Aggregation has also been mentioned in other studies on G-CSF secretion from *P. pastoris* and *S. cerevisiae*.[40,37,38] It appears that aggregation is also not uncommon for other secreted heterologous proteins produced in yeast and fungal hosts, and that this depends on intracellular and extracellular stress factors.[47–50,28] Expression of G-CSF in the bacterium *E. coli* is somehow paradoxical to the results for yeast and fungi, in our experience. Although G-CSF is expressed in the form of inclusion bodies, the induction/ fermentation process can be conducted in a specific way to obtain particular, 'non-classical' inclusion bodies that contain large amounts of the correctly folded protein and protein precursor, respectively. Such inclusion bodies show high solubility and can thus be extracted and purified under mild, non-denaturing conditions.[12,51,52]

4. 5. Glycosylation of Recombinant G-CSF

Due to its size of ~19 kDa on SDS-PAGE gels, the G-CSF produced in *A. niger* appeared not to be glycosylated, which was also confirmed using glycoprotein detection kits. Heavy glycosylation was not expected, as there are no N-glycosylation sites in the primary structure of G-CSF. There is normally *O*-glycosylation on exposed Ser or Thr amino-acid residues, although natural human G-CSF and recombinant G-CSF produced by mammalian cells have only a short carbohydrate chain attached to Thr133, which is known as a high potential site in higher eukaryotes.[53–54] Fungi glycosylate proteins in a fashion similar to higher eukaryotes, and therefore some O-glycosylation was expected, although a comparable situation, namely essentially non-glycosylated protein, was also reported for the expression/ secretion of G-CSF in *P. pastoris* and *S. cerevisiae*.[46,37,38]

4. 6. Folding and Biological Activity of the Isolated Recombinant G-CSF

Immobilised metal affinity chromatography was used as an analytical tool to determine the G-CSF folding/ renaturation efficiency during the SEC. It has been reported previously that only the correctly folded biologically active monomer forms of G-CSF will selectively bind to an IMAC support under native conditions, whereby the incorrectly folded or aggregated molecules of G-CSF will elute in the flow-through fractions, without being retained.[51] Indeed, only in the correctly folded monomeric G-CSF molecules do the naturally present histidine residues have the specific distribution and orientation that promotes their strong binding to IMAC matrices via coordinate bonding.[51,55]

Our *in vitro* biological testing showed that the majority of the secreted recombinant G-CSF present in this *A. niger* native filtrate was essentially biologically inactive, as it had <10% of the expected proliferative activity. The reason for this low activity could lie in the incorrectly folded molecule and/or in the aggregated recombinant protein or an absent or incorrect glycosylation pattern. G-CSF is a hydrophobic protein by itself, although this recombinant G-CSF from *A. niger* most probably contained additional exposed hydrophobic regions due to the incompletely folded molecules. Fully biologically active protein is only obtained with the correctly folded monomeric molecules, which according to *in vitro* biological activity testing, represented <10% of the secreted G-CSF. In numerous proteins, absent or incorrect glycosylation patterns can also negatively affect the biological activity. Although it has been reported that oligosaccharide units on Thr133 of CHO-cell-derived G-CSF, which is identical to the endogenous protein, can reduce the protein flexibility locally to allow its improved interaction with the receptor, it is known that in the case of G-CSF, glycosylation is not essential for biological activity.[56] The *E. coli* derived G-CSF protein without any glycosylation shows full biological activity. However, as this form of G-CSF has no sugar 'shield', it is more susceptible to environmental factors and prone to aggregation, and hence it is difficult to formulate. This certainly also holds for this recombinant G-CSF from *A. niger*, which had no attached mannose molecules. The *in vitro* biological activity of this G-CSF from *A. niger* when it was SEC purified and renatured was considerably increased, which clearly shows the effectiveness of the approach used. However, to obtain the fully active protein, further chromatographic purification or optimisation of the denaturation/ renaturation procedure is needed; e.g., a higher concentration of the denaturing agent, or use of ot-

her denaturing agents or classical renaturation/denaturation procedures via dilution. Also, an unpaired cysteine residue, or here a cysteine-mutated glycosylation site at Thr133, can be PEGylated to improve the therapeutic properties of protein drugs, and to resolve poor solubility, immunogenicity and stability issues.[57]

5. Conclusions

The high capacity for the secretion of homologous proteins of filamentous fungi represents a promising expression/ secretion system for heterologous proteins, and this is the first report on the expression of the medically important G-CSF in *A. niger*. The majority of the *A. niger* secreted G-CSF was in the form of soluble aggregates and showed <10% of the expected proliferative activity. After applying a denaturing concentration step and single-step chromatographic separation with simultaneous renaturation, the G-CSF proliferation activity increased considerably (up to 65% of that expected). This coincided with the G-CSF protein being in the monomeric and correctly folded form, as confirmed by analytical IMAC. Fungi still hold promise for expressing pharmaceutical proteins, although several attempts have already been made in *A. niger*, as for human interleukin 6, non-glycosylated proteins like TNFα and most successfully for different antibodies.[30,58–61]

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Povzetek

Prvič smo opisali sistem za izražanje in izločanje medicinsko pomembne človeške beljakovine G-CSF iz glive *Aspergillus niger*. Izbrali smo zanesljiv način izražanja s fuzijo beljakovine G-CSF v skupnem bralnem okviru za encimom glukoamilazo, ki ga gliva v veliki količini izloča, in cepitvenim mestom KEX2. To je omogočilo pridobitev 5–10 mg/l gojišča pravilno odcepljene beljakovine G-CSF, vendar glavnina (>90 %) ni bila biološko aktivna. Po koraku denaturacije/koncentracije in kromatografski ločbi/renaturaciji se je proliferacijska aktivnost beljakovine G-CSF znatno povečala in analiza s kovinsko-kelatno afinitetno kromatografijo je potrdila monomerno in pravilno zvito beljakovino. Ti podatki kažejo, da se je ta človeška beljakovina izločila nepravilno zvita v gojišče glive *A. niger* in da je ušla kontrolnemu mehanizmu pravilnega zvijanja beljakovin v endoplazemskem retikulumu. Našo ugotovitev smo obravnavali v primerjavi z zvitjem beljakovine G-CSF, proizvedene v bakterijah in kvasovkah.