A TNF receptor antagonistic scFv, which is not secreted in mammalian cells, is expressed as a soluble mono- and bivalent scFv derivative in insect cells

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Abstract

Single chain antibodies (scFv) are usually produced in E. coli, but generation of certain scFv derivatives, such as complex fusion proteins or glycosylated forms of scFv is restricted to eukaryotic expression systems. We investigated the production of soluble mono- and bivalent single chain antibodies (scFv) in eukaryotic cells and describe a cassette vector system for mammalian and baculovirus expression which is compatible with an established vector system for bacterial expression and phage display selection of scFvs. The applied model scFv was derived from a murine antibody (H398) against human tumor necrosis factor receptor 1 (TNFR60), known to be a potent antagonist of TNF action in its monomeric form and a potential therapeutic agent for treatment of TNF-mediated diseases. Surprisingly, the monomeric scFv form of H398 (scFv H398) is expressed but not secreted in different mammalian cells. In contrast, in insect cells using recombinant baculovirus, a monovalent scFv H398 and a bivalent scFv fusion protein with an human IgG1 Fc region were expressed and secreted with correctly processed signal sequence. Concerning the influence of valency of the model Ab and its derivatives on antigen binding affinity and neutralisation of TNF activity, we found that the mono- and bivalent form of scFv H398 possesses the same characteristics as proteolytically produced Fab H398 and original mAb H398, respectively. Furthermore, fusion of the Ig Fc protein to scFv H398 increase the in vitro half-life at 37°C. We conclude that the described cassette vectors readily allow the eukaryotic expression of mono- and bivalent scFv derivatives to analyse the influence of valency of scFv molecules on antigen binding and biological activity. © 1997 Elsevier Science B.V.

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1. Introduction

In recent years, advances in antibody (Ab) technology have led to the development of single chain antibody fragments (scFv) which are constructed from monoclonal antibodies (mAb) of hybridoma cells or isolated from scFv phage display libraries. These recombinant molecules possess a high clinical potential as they open new therapeutic and diagnostic strategies [1] due to several unique features, which become apparent from a variety of in vitro and in vivo applications, such as superior tissue penetration and rapid blood clearance via the kidney [2,3]. Concerning the biotechnological production, the use of scFv molecules circumvents complications associated with efficient heteromer formation of VH and VL. Moreover, scFv expressed as fusion proteins with immunoglobulin Fc region led to efficient formation of homodimeric divalent molecules, which exert binding avidity and effector functions comparable to those of complete Ab [4].

Most scFvs can be efficiently produced in E. coli either as soluble proteins, secreted into the periplasm, or as insoluble cytoplasmic inclusion bodies, which require in vitro refolding for isolation of functional Ab fragments [5]. However, for some scFv both approaches may result in poor yields of functional Ab fragments [5,6]. In search of alternative sources for soluble, functional scFv, mammalian cell expression systems have been applied in order to use the enzyme and chaperone-assisted folding mechanism of the endoplasmic reticulum and the secretory pathways of the golgi apparatus of the higher cells [7-9]. A further conceptual advantage of eukaryotic expression systems is that they are capable of post-translational modifications, e.g. glycosylations, and of producing and secreting structurally complex and large derivatives of scFv-like homodimeric scFv Ig Fc fusion proteins [4,7,10]. Nevertheless, some scFvs have been reported to be refractory to expression or secretion in mammalian cells [8,9,11].

The aim of our study was to establish an eukaryotic expression system, which allows expression and secretion of scFvs as mono- and bivalent antigen binding molecules. We, therefore, investigated production of soluble scFvs in mammalian and insect cells. The rationale of our approach was to provide a system for the evaluation of the effect of mono- and bivalency of scFvs on affinity/avidity in antigen binding as well as in biological assays. This is, for example, of fundamental importance for development of cytokine receptor specific Abs. Some of these Abs are potent receptor agonists in their natural, bivalent form by mimicking ligand dependent receptor dimerization. Provided these antibodies are competitors of ligand binding, they can be readily converted into effective antagonists when expressed as or processed into a monovalent Fab or scFv form [12,13].

The model Ab in the present investigation is a murine Ab (H398) against the human TNF receptor 1 (TNFR60) which, in particular, in its monomeric Fab form [14] and as bacterially produced scFv, is a potent antagonist of TNF activity [6]. As TNF is known to be a pathogenic factor in several autoimmune and inflammatory diseases, e.g. endotoxic shock [15], graft vs. host disease [16], cachexia [17], rheumatoid arthritis [18] and Crohn’s disease [19], the development of novel antagonists with clinical potential is of great importance.

Besides scFv expression in mammalian cells (see cited reports above) examples which prove, in principle, the feasibility of the baculovirus system for expression of complete Abs and derivatives thereof have been described [20,21]. In our present study we have developed a cassette vector for scFv expression in mammalian cells as well as two novel baculovirus transfer vectors which contain exchangeable scFv cassettes for expression of monovalent scFv and bivalent scFv Fc fusion molecules in insect cells. All vectors were designed to be compatible with primer and vector systems frequently applied for cloning and selection of defined scFvs in bacteria. Using these novel cassette vectors, we describe here that a TNFR60 specific Ab fragment is expressed and secreted in Sf 158 as a functional mono- and bivalent scFv. As this scFv accumulates intracellularly but is not secreted in various analysed mammalian cells the described baculovirus system appears appropriate to overcome this problem in eukaryotic scFv production.
2. Materials and methods

2.1. Vector construction

The cloning vector (pLOPP) was constructed to allow the generation of an eukaryotic scFv expression cassette which contains a eukaryotic signal sequence and which is compatible, as regards reading frame and restriction endonuclease site with the bacterial expression vectors, pOPE and phage display vectors, pSEX [22]. The signal sequence used originated from the human IL-6 receptor (IL-6 R) and was isolated by PCR from IL-6 R cDNA [23] using the oligonucleotides # 1 and # 2. Thereby introducing the endonuclease restriction sites XhoI at the 5' end and PvuII at the 3' end of the amplified DNA sequence. The IL-6 R signal sequence DNA was cloned into the bacterial expression vector pOPE50 via XhoI and PvuII and replaced the bacterial leader sequence. Derivatives of pLOPP with scFvs encoding sequences of the murine mAb H398 specific for human TNFR 60 [6] and of the murine mAb YII/2 specific for tubulin [24] were cloned using already existing scFv constructs. For construction of pMAM/scFv H398 and pMAM/scFv YII/2 the complete eukaryotic scFv expression cassette was cut from pLOPP using XhoI and an XhoI site (located downstream of the scFv cassette) and cloned into the expression vector for mammalian cells, pCDM8 (Invitrogen, Leek, The Netherlands). Alternatively, the eukaryotic scFv expression cassette from pLOPP was amplified by PCR using primer # 1 and # 3, the latter introducing an XhoI site into the 3' end of the DNA fragment, and finally cloned into pCDM8 as a XhoI fragment. For construction of the transfer vector pBV/scFv, in order to generate recombinant baculovirus for scFv expression in insect cells, the complete eukaryotic scFv expression cassette from pLOPP/scFv H398 was used. Vector pLOPP/scFv H398 was cut with XhoI and treated with Sequenase DNA polymerase (Amersham Buchler, Braunschweig, Germany) in the presence of dNTP to generate DNA blunt ends. After cutting with XbaI, the scFv expression cassette was isolated and cloned into baculovirus transfer vector pVL1393 (Dianova, Hamburg) prepared previously with Smal and XbaI. To construct the baculovirus transfer vector pBV/scFv-Fc for expression of bivalent scFv proteins, a transfer vector (pVL1393-Fc) containing the DNA sequence of human IgG1 Fc (hinge, CH2, CH3) was used which has been described before for production of cytokine immunoconjugates [25,26]. The complete scFv H398 expression cassette from pBV/scFv H398 was excised as a BamHI fragment and inserted in the correct orientation, upstream of the Fc encoding region into pVL1393-Fc.

Applied oligonucleotides:
1. 5'-GATCCTCGAGCCATGCTGGCGTCGGCC-3';
2. 5'-GATCCAGCTGAACTTGCGCCGCTCCCGGGCG-3';
3. 5'-GATCCTCGAGCGGCCGCCTCAAGCTAGCTGATCA-3'.

2.2. Transient expression of recombinant Ab in mammalian cells

The cell lines BHK-21 (baby hamster kidney cells) and 293 (transformed primary human embryonal kidney cells) were cultured in Dulbecco's modified Eagle's medium and COS-7 cells (monkey kidney fibroblasts) with RPMI medium at 37°C whereby all media were supplemented with 10% heat inactivated fetal calf serum (FCS). Cells with a density of 2 x 10^5 per well were seeded in a 6-well plate and transfected 16 h later with 3 µg vector DNA using Lipofectamin (Gibco BRL, Eggenstein, Germany) according to the protocol of the manufacturer. After transfection the cell cultures were cultivated in medium with FCS for 36 h and finally analysed for expression and secretion of scFv. For detection of intracellular scFv the cells were washed with PBS (phosphate-buffered saline: 120 mM NaCl, 17 mM Na2HPO4, 3 mM KH2PO4, pH 7.2) and lysed on ice in a buffer containing 140 mM NaCl, 10 mM Tris-HCl, 2 mM MgCl2, 1 mM DTT, 2 mM PMSF, 0.5% (v/v) Nonidet-P40. After centrifugation (14 000 x g, 15 min) the soluble lysate fraction was analysed.
2.3. Production of recombinant baculoviruses

Recombinant BV were generated by in vivo recombination between BV DNA and transfer vector DNA using modified linearized AcNPV DNA (Baculo Gold DNA, Pharmingen, Hamburg) as described [25].

2.4. Production of scFv derivatives in insect cells

For maximum expression of functional recombinant antibodies in Sf 158 cells antigen binding studies were performed to determine the optimum in multiplicity of infection and time course of cell cultivation. Based on these investigations cell cultures for production of recombinant Ab were incubated until 4 days post-infection. The culture supernatant containing the recombinant protein was collected, ultracentrifuged (100 000 × g) and used for analysis and purification of the recombinant Ab.

2.5. Chromatography of mono- and bivalent scFv

For Ni-chelate chromatography of scFv H398 the following procedure was applied: Sf 158 culture supernatant was adjusted to 1 mM PMSF (phenylmethylsulfonylfluoride), 3 mM sodium azide, ultracentrifuged (100 000 × g) for 1 h and dialysed 16 h against a buffer containing PBS adjusted to pH 8. Finally the solution was adjusted to 0.1% Triton X-100, 0.5 M betaine (pH 8) and 5 mM imidazol. Recombinant Ab was immobilized with Ni QIA express (Qiagen Hilden, Germany; 1/50 volume of the original sample volume) by incubation under continuous shaking at 4°C for 2 h. The Ni-NTA matrix was carefully pelleted by centrifugation (300 × g) and sequentially washed with the following buffers (quantities refer to the volume of the pelleted matrix): 5 volumes of washing buffer [0.5 M betaine, 10% glycerol, 40 mM Na phosphate (pH 8)] containing 10 mM imidazole, 5 volumes of washing buffer containing 20 mM imidazole. Finally the Ni-NTA matrix was washed with washing buffer (10 volumes), transferred to a column and scFv H398 was eluted with washing buffer containing 200 mM imidazol and fractionated. For purification of scFv H398-Fc protein A chromatography was used. Sf 158 culture supernatant was ultracentrifugated and passed over a protein A column (Hi trap protein A, Pharmacia, Freiburg, Germany, 1 ml matrix) at a flow rate of 0.5 ml per minute. The column was washed with 20 ml PBS and finally eluted with PBS adjusted to pH 3. The pH of the elution fractions was immediately neutralized with 0.4 M Na phosphate, pH 9.

2.6. Analyses of scFv derivatives by SDS-PAGE and immunoblot

Culture supernatant, cell extracts or chromatography samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE). The protein bands in the gel were visualized by silver staining or transferred to nitrocellulose membrane (Macherey and Nagel, Germany) using a semidry blotting apparatus (Phase, Germany). The nitrocellulose membranes were incubated with a c-myc specific monoclonal Ab or a specific rabbit serum which only recognises the processed aminoterminus of scFv [27]. Subsequently an alkaline phosphatase-conjugated second Ab (Bio-Rad, Munich, Germany) was applied to detect the scFv/Ab complex using NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) as enzyme substrate.

2.7. Determination of the dissociation constant of scFv H398

Affinity constants of the recombinant and natural Ab fragment for antigen in solution were determined as described [28]. Briefly, constant amounts of scFv H398 (0.03 µg/ml) or natural Fab H398 (0.05 µg/ml) were incubated with various concentrations of recombinant TNFR60 in the range of 0.32–40 nM for 2 h at room temperature. The proportion of free Ab was detected by ELISA with immobilized antigen using an incubation time of 20 min. Coating of antigen was done with 10 ng/well in PBS. All samples were in triplicate. The dissociation constant (K_d) was calculated from the binding saturation curve.
2.8. Antigen binding competition studies

To compare antigen binding of scFv H398, scFv H398-Fc and mAb H398 binding competition studies with Fab H398 were performed. First, 1 nM recombinant Ab or mAb H398 were mixed with various concentrations of Fab H398 in PBS containing 2% bovine serum albumin and finally incubated 2 h at room temperature in a volume of 50 µl in the wells of microtiter plates with immobilized TNFR60. (Coating of the microtiter plates was done with 50 ng/50 µl TNFR60 per well at 4°C for 16 h). Bound scFv H398 was detected with a c-myc specific mAb followed by an anti-murine IgG-specific goat Ab-conjugated with alkaline phosphatase. For detection of scFv H398-Fc and mAb H398, alkaline phosphatase-conjugated goat Abs against human and murine IgG Fc were used, respectively. p-Nitrophenyl phosphate was applied as a substrate for the calorimetric reaction induced by alkaline phosphatase.

2.9. Determination of the temperature stability of scFv

Affinity purified scFv H398 or scFv H398-Fc (5 µg/ml) was incubated at 37°C in culture medium containing 5% fetal calf serum. Aliquots were taken sequentially at various times and analysed for antigen binding activity by ELISA. The relative binding activity of the incubated samples was calculated using recombinant Ab stored at 4°C as a reference.

2.10. Assay for competition of TNF cytotoxicity

In principle this assay was carried out as previously described [6]. KYM-1 cells were plated in microtiter plates at a density of 1 x 10^4 cells/well and incubated for 16 h for adhesion of the cells. Thereafter, the KYM-1 cells were incubated for 24 h with TNF (250 pg/ml, BASF, Germany) in the presence of various concentrations of scFv H398, scFv H398-Fc, Fab H398 or original H398. To detect cell viability, the metabolic activity of the cultures was assayed by incubation of the wells with MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), 50 µg/well) for 2 h at 37°C. Subsequently, cells were lysed by adding 10 µl of a 20% SDS solution in 0.02 HCl and after incubation for 4 h at room temperature the optical density of the lysate was determined at 560 nm. All samples were assayed in triplicate. The efficiency of H398 and its derivatives to neutralise TNF bioactivity was expressed as percent protection whereby cells cultured in the presence of TNF without antagonists and cells grown only in media were used to calculate 0 and 100% protection, respectively.

3. Results

For the expression of scFv fragments in mammalian and insect cells, the mammalian cell expression vector pMAM/scFv and the baculovirus (BV) transfer vector pBV/scFv were constructed (Fig. 1). Both vectors contain the same scFv expression cassettes in which the reading frame of the scFv encodes a eukaryotic signal sequence, a C-terminally fused c-myc domain and a histidine heptamer for secretion, detection and purification of the recombinant protein, respectively. The scFv expression cassette is under transcriptional control of the CMV promoter in pMAM/scFv and the polyhedrin promoter in pBV/scFv. The restrictions sites for cloning of VH and VL encoding sequences are compatible with those of vectors described recently for cloning and bacterial expression of scFv [22,29]. The VH-linker-VL encoding sequences of the murine TNFR 60-specific single chain antibody, scFv H398, were cloned into pMAM/scFv and pBV/scFv, to construct pMAM/scFv H398 and pBV/scFv H398, respectively. In addition, scFv Y11/2 which was cloned from a murine hybridoma specific for human tubulin and used here for control purposes was also cloned in pMAM/scFv, resulting in pMAM/scFv Y11/2. Furthermore, a transfer vector was constructed for expression of covalently linked homodimeric and therefore bivalent scFv fusion proteins. For this purpose the scFv H398 sequence was fused to the 5'-end of a human IgGl Fc encoding sequence giving rise to pBV/scFv H398-Fc.
3.1. Different mammalian cells do not secrete monovalent scFv H398

Vector MAM/scFv H398 was transfected into BHK-21 and COS-7 cells and analysed by immunoblotting for expression and secretion of scFv. scFv H398, with an apparent molecular weight of 30 kDa was detected as a single band in the cell pellet but not in the supernatant of pMAM/scFv H398 transfected BHK-21 cells (Fig.
2). Additional experiments with pMAM/scFv H398 transfected in COS-7 and 293 cells confirmed the failure of secretion of scFv H398 in mammalian cells (data not shown). In contrast, when COS-7 cells transfected with pMAM/scFv Y11/2 were used, the recombinant Ab fragment, scFv Y11/2, was detected in the supernatant as well as in the cell pellet. The signal sequences of the secreted scFv Y11/2 was correctly cleaved off as shown by immunoblot analyses with an antisem (serum A) which only recognises the processed N-terminus of the scFv protein [27], thereby indicating that the applied mammalian expression system is, in principle, functional (Fig. 3).

3.2. Expression and secretion of mono- and bivalent scFv H398 in insect cells and their isolation from cell culture supernatant

The transfer vectors pBV/scFv H398 and pBV/scFv H398-Fc were used to generate recombinant BV for expression of mono- and bivalent scFv forms. After infection of Sf 158 cells with the respective recombinant BV, scFv H398 and scFv H398-Fc were detected by immunoblot analyses in the culture supernatant with apparent molecular weights of 30 and 130 kDa, respectively, thus

Fig. 2. Immunoblot analyses of transient scFv H398 expression with BHK-21 cells. BHK-21 cells were transfected with pMAM/scFv H398 (lane 1 and 3) or with pMAM/control (a vector without functional scFv reading frame). Then, 36 h after transfection soluble fractions of cell lysates (lane 1 and 2) and culture supernatants (lane 3 and 4) were separated by SDS-PAGE (12%) and analysed as a immunoblot for the presence of scFv using a c-myc specific mAb.

revealing expression and secretion of the recombinant scFv derivatives (Figs. 4 and 5). The yields of functional scFv H398 and scFv H398-Fc were about 200 and 600 µg per litre culture, respectively. The signal sequences of these recombinant proteins were correctly processed upon secretion as shown by immunoblot analyses with serum A (Figs. 4 and 5). Further, immunoblot analyses of scFv H398-Fc under reducing and non reducing conditions indicated its covalently linked homo-
Fig. 5. Expression and signal sequence processing of scFv H398-Fc in insect cells and its purification. Recombinant baculovirus were generated with the scFv cassette transfer vector pBV/scFv H398-Fc and used for infection of Sf 158 cells (lane 1, 3 and 5). In addition, Sf 158 cells were infected with a recombinant baculovirus expressing a irrelevant protein (lane 2 and 4). Four days post-infection supernatant of the infected cells (lane 1–5) or purified scFv H398-Fc (lane 6 and 7) was separated by SDS-PAGE (10%) under non-reduced (lane 1, 2, 6 and 7) or reduced conditions (lane 3–5). Immunoblot analysis with a c-myc specific mAb (lane 1–3 and 6) was used to detect scFv H398-Fc or serum A to prove signal processing (lane 4 and 5). Additionally purified scFv H398 was silver stained after SDS-PAGE (lane 7).

Fig. 6. Stability of purified scFv H398 and scFv H398-Fc at 37°C. Recombinant Ab were incubated at 37°C and antigen-binding was monitored by ELISA at various times of incubation.

The antigen binding affinity of the scFv H398 from Sf 158 cells after chromatography was analysed in solution by ELISA as described by Friguet et al. [28] and compared with a bacterially expressed scFv H398. The two monovalent scFv constructs exerted an affinity with a $K_d$ of 1 nM, as calculated from the saturation curves (Fig. 7) which is similar to the $K_d$ (1.5 nM) of the natural Fab H398 [6]. Due to the higher affinity of the bivalent scFv, the method of Friguet et al. was not suitable to determine its exact antigen affinity. Hence additional binding competition studies with natural Fab H398 were performed to compare the binding affinity of scFv H398 and scFv H398-Fc. The corresponding data in Fig. 8 show that mAb H398 and scFv H398-Fc were displaced by Fab H398 at a $IC_{50}$ of 89.2 nM and 71 nM, respectively. In contrast, an about 3–4-fold lower concentration of the Fab H398 (IC$_{50}$ 22 nM) was needed for competition of scFv H398.

The monovalent forms of H398 have been described as potent TNF receptor antagonists in vitro [6,30], whereas complete H398 may possess in some experimental models at high concentrations a limited agonistic activity, probably due to partial receptor cross linking and activation ([31]; Moosmayer, unpublished data). Therefore, we in-
investigated whether mono- and bivalent scFv H398 forms differ in their capacity to protect KYM-1 cells from cytotoxicity induced by soluble TNF. KYM-1 cells were incubated with purified recombinant antibodies, original H398 or natural Fab H398 in the presence or absence of TNF and the cell viability was assayed after 24 h. TNF mediated cytotoxicity is completely inhibited by scFv H398 with a EC₅₀ (half maximum effective concentration) of 20 nM, which is close to the EC₅₀ of 8.2 nM determined for the natural Fab H398 in a parallel assay (Fig. 9). Bivalent scFv H398-Fc also inhibited TNF-mediated cytotoxicity, but without reaching full protection. This is reminiscent of the incomplete neutralisation capacity of the natural bivalent H398 in the same bioassay (Fig. 9).

4. Discussion

We describe here two cassette transfer vectors for generation of recombinant baculoviruses to express mono- and bivalent forms of scFvs in insect cells. In addition, a cassette expression vector for scFv expression in mammalian cells was developed and applied. All vectors are designed to be compatible with primer sets recently described.
for the cloning of scFv repertoires and scFvs from hybridoma [29] as well as with the bacterial expression vector pOPE and the phage display vectors pSEX [22], thereby completing a well established system for selection, production and characterisation of scFvs with defined specificities. In order to use the scFv expression cassettes of this bacterial cloning and selection system for eukaryotic expression of secretory scFvs, a heterologous eukaryotic signal sequence, originating from human IL-6 receptor, was provided in the eukaryotic expression vectors described here.

We show that scFv H398 and the homodimeric scFv H398-Fc are produced as secretory proteins with the expected molecular weights in insect cells. Both scFv derivatives were found to be correctly processed, demonstrating that the non-Ig-signal is functional in combination with the Ig V sequences. In addition, we successfully used this heterologous signal sequence with a panel of other scFvs in eukaryotic cells giving evidence for a broad utility of this signal peptide (data not shown).

The secretion of biologically active scFv H398 in insect cells is of relevance, as this particular scFv was not secreted in any of the investigated mammalian cell lines (COS-7, BHK-21, 293), which are the standard cell lines in many eukaryotic expression systems. This failure in secretion in mammalian cells is very likely due to the V regions of H398, as this effect also occurred when the original H398 VH signal sequence was used (data not shown), but was not observed with a scFv of different specificity using the same expression system. Nevertheless, the scFv cassette expression vector MAM/scFv has successfully been used for transient and stable expression and secretion of different functional scFvs (D. Moosmayer, unpublished data). Different examples of scFvs which are refractory to production or secretion in mammalian cell have been described before [8,9,11]. Such failures in secretion of scFvs in mammalian cells were reported to occur independently irrespective of whether the original signal sequence of the corresponding V region or a heterologous Ig signal were used [8,11]. In one instance of a scFv with impaired secretion in mammalian cells the exit from the endoplasmic reticulum was identified as the limiting step. This defect could be overcome by addition of a glycosylation site into the VH region [8]. Differences in the secretory mechanism of mammalian and insect cells can also affect secretion of complete Ab heavy chains and have been related to a differential function of BiP (binding protein) in these two species [32]. BiP interaction with a non-secreted scFv in mammalian cells has been reported, too, and might be a mechanism involved in retention and/or secretion of scFvs [11].

The amounts of H398 scFv derivates produced with the described expression system were sufficient for analytical purposes and can easily be scaled up in stirring flasks. The expression yield of secreted, functional scFv H398 in insect cells (200–600 µg/l) is comparable to our previous yields (400 µg/l bioactive material) obtained from expression of the same scFv in E. coli [6] as inclusion bodies but circumvents the cumbersome in vitro refolding procedure. Accordingly, the described approach appears to be an efficient alternative to prokaryotic expression. More recent studies performed by us support this notion. We successfully applied the described cassette transfer vectors for production of mono- and bivalent tetanus toxoid-specific human scFvs with about ten-fold higher expression yields (3–5 mg/l, Moosmayer, unpublished results), suggesting an influence of the V regions sequences on the expression capacity in the described cell/vector system.

With regard to the isolation of the recombinant scFv from cell culture supernatants, we have applied convenient one step chromatography procedures, which give readily access to material suitable for analytical purposes. We found that Protein A chromatography of scFv-fusion proteins was much more efficient than the Nickelate chromatography of monovalent scFvs.

Functional analyses revealed that monovalent scFv H398 from insect cells possesses the expected antigen binding affinity and TNF-neutralization capacity almost identical to those values obtained with bacterially expressed scFv H398 and natural Fab H398 [6,30]. Dimerization of scFv H398 via an Ig Fc region resulted in a fusion protein with an antigen binding characteristic comparable to
the natural bivalent mAb H398, as revealed by binding competition studies. Moreover, dimerization of scFv H398 led to an increase in its stability at 37°C which could be important for the in vivo half-life of the recombinant molecule. In short term bioassays (24 h), scFv H398-Fc showed a similar characteristic in neutralization of TNF activity as recently described for parental mAb H398 [6], although higher concentrations were required for the former. Both reagents exerted an incomplete protection from TNF activity at high Ab concentrations, suggesting that the bivalent molecules may possess a limited agonistic activity due to partial TNFR60 cross linking; an observation which has been made previously with mAb H398 ([31]; Moosmayer, unpublished data).

In conclusion, our results demonstrate that for scFv production the baculovirus expression system in combination with our cassette vectors is an effective alternative to prokaryotic and mammalian expression systems. First, it allows the generation of mono- and bivalent forms of scFv which are suitable to investigate the influence of the mono-/bivalency of Ab molecules upon antigen binding as well as their biological activity. Second, the baculovirus expression system is capable of secreting a scFv which is refractory to secretion in various mammalian cells, suggesting differences in the scFv secretion pathways in the cell lines of both species. It will need further investigations as to whether this approach is generally applicable to other scFvs with impaired secretion in mammalian cells.

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References


