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Tumor-directed lymphocyte-activating cytokines: refolding-based preparation of recombinant human interleukin-12 and an antibody variable domain-fused protein by additive-introduced stepwise dialysis☆,☆☆

Koki Makabe[a], Ryutaro Asano[a,b], Takahiko Ito[a], Kouhei Tsumoto[a], Toshio Kudo[b], Izumi Kumagai[a,*]

[a] *Department of Biomolecular Engineering, Graduate School of Engineering, Tohoku University, Aoba-yama 07, Aoba-ku, Sendai 980-8579, Japan*
[b] *Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University, 4-1 Seiryomachi, Aoba-ku, Sendai 980-8575, Japan*

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Abstract

Integration of lymphocyte-activating cytokines (e.g., interleukin-12: IL-12) to tumor cells offers promise for cancer immunotherapy, but the preparation of such heterodimeric proteins by refolding is difficult because of subunit instability. We achieved the refolding of *Escherichia coli*-expressed human IL-12 by a stepwise dialysis method, preventing the formation of insoluble aggregates by adding a redox reagent and an aggregation suppressor. We also constructed a tumor-specific IL-12 protein, each subunit of which was fused with one chain of variable domain fragment (Fv) of anticarcinoembryonic antigen (CEA) antibody T84.66 (aCEA-IL12). Fusion of IL-12 with Fv greatly increased the yield of functional heterodimer. Several assays have indicated that the Fv domain and IL-12 domain of the fused protein had cognate biological activities, and it enhanced the cytotoxicity of T-LAK cells for the cancer cell line.

Keywords: Interleukin-12; Cancer immunotherapy; Refolding; Fusion protein; Fv

Recent advances in genetic engineering have enabled us to prepare various recombinant proteins, e.g., cytokines, costimulatory molecules, antibody fragments, and other functional molecules [1–4]. Indeed, successful construction of several proteins by using bacterial expression systems has been reported, and some of these

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☆☆ *Abbreviations:* AISD, additive-introduced stepwise dialysis; β-ME, β-mercaptoethanol; BrdU, 5-bromo-2′-deoxyuridine; CEA, carcinoembryonic antigen; ELISA, enzyme-linked immunosorbent assay; E:T, effector:target ratio; FITC, fluorescein isothiocyanate; Fv, fragment of immunoglobulin variable regions; GdnHCl, guanidium hydrochloride; GSH, glutathione with reduced form; GSSG, glutathione with oxidized form; IMAC, immobilized metal affinity chromatography; IFN, interferon; IPTG, isopropyl-1-thio-β-D-galactopyranoside; T-LAK, lymphokine activated killer with T cell phenotype; mAb, monoclonal antibody; MTS, 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cells; rIL-2, recombinant interleukin-2; rIL-12, recombinant interleukin-12; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

* Corresponding author. Fax: +81 22 217 6164.
E-mail address: kmiz@mail.tains.tohoku.ac.jp (I. Kumagai)

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proteins are now used for therapeutic purposes [5–8]. Overproduction of proteins in *Escherichia coli*, however, often leads to the formation of insoluble aggregates, referred to as inclusion bodies, in the cytoplasmic or periplasmic space [9]. In many cases, the major components of these inclusion bodies are expressed proteins, and, once refolding has been successfully performed, large amounts of functionally active protein can be made available to meet industrial and therapeutic demands [10–15]. Therefore, a stable and convenient refolding system would be useful for the preparation of recombinant proteins.

Many proteins with heteromultimeric structures (composed of more than two subunits) are hard to prepare owing to the instability of some domains. Oligomerization is usually performed through interfacial interaction [16,17], in which subunits cooperatively interact with each other in several ways, e.g., by domain–swapping and coiled–coil association. These observations suggest that the distinctly different folding properties among subunits within a protein lead to improper folding of the protein during the in vitro folding process.

The heterodimeric cytokine interleukin-12 (IL-12) consists of two subunits, p35 and p40, which are connected by disulfide bonds via surface cysteine residues [18,19]. IL-12 is involved in the regulation of both innate and adaptive immunity; it has pleiotropic effects on T cells, B cells, and natural killer cells, and functions as a key regulator of Th1 cell differentiation [20–22]. Several clinical trials have been conducted to evaluate the therapeutic potential of IL-12 [23–26] and promise to be useful in various therapeutic procedures. However, practical use of this cytokine for various purposes has been limited owing to the instability of the p35 subunit and the stable formation of an antagonistic p40 dimer [26]. Several reports have demonstrated improved preparation of human IL-12, e.g., by the formation of single-chain IL-12 [27] and by DNA shuffling [28].

We recently reported a high-yield refolding process of single-chain antibody and other proteins by immunoglobulin folding [29], and of the four-helix-bundle-type cytokine human IL-21 [30]. The p35 subunit is classified as a four-helix-bundle cytokine and the p40 subunit contains the immunoglobulin fold. If disulfide bond formation is a critical step for correct folding of the protein, then the dialysis method developed for refolding of immunoglobulin-like proteins may be used for heterodimeric proteins if some parameters (e.g., concentration of the redox solution) are correctly adjusted. In addition, cooperative refolding of the two subunits may overcome both the aggregation of p35 due to instability and p40 dimer formation.

Here, we describe the successful reconstruction of a heterodimeric protein, refolded IL-12, and a tumor-specific IL-12 protein fused with a carcinoembryonic antigen (CEA)-specific antibody variable region (Fv), designated as aCEA-IL12 (Fig. 1), by using an additive-induced stepwise dialysis (AISD) method. Mouse monoclonal antibody T84.66 IgG was used as an anti-CEA antibody [31–33]. Some studies indicate that the use of antibody fused with IL-12 as a cancer drug is superior to that of IL-12 alone [34–36]. The refolded IL-12 and aCEA-IL12 prepared were found to have var-

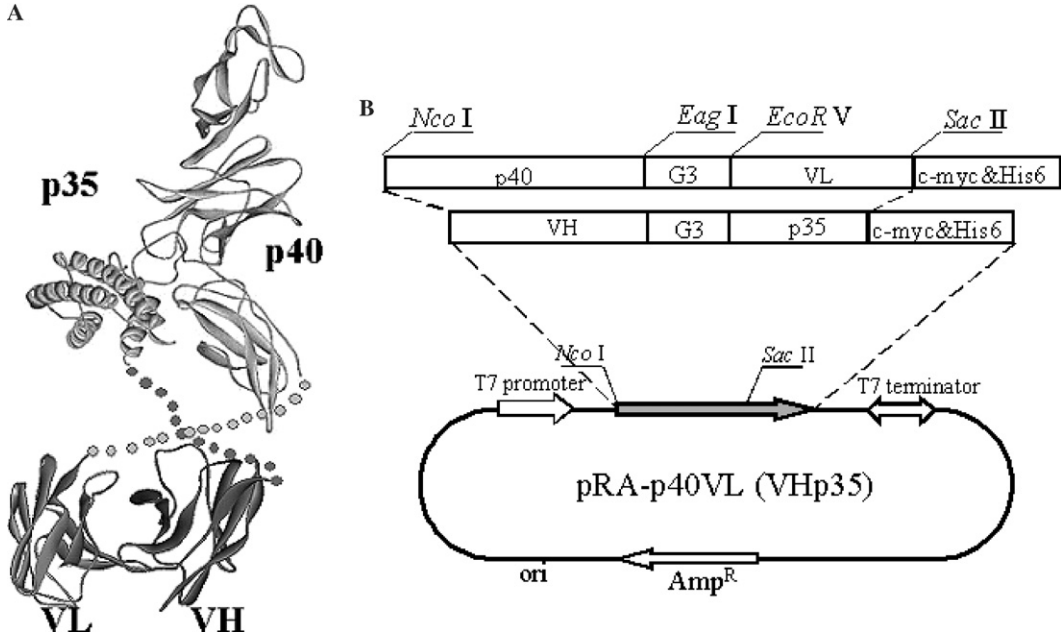

Fig. 1. Design of aCEA-IL12. (A) Schematic representation of aCEA-IL12, based on the crystal structure of human IL-12 [11] and the Fv region of anti-HEL antibody, HyHEL-10 [41]. (B) Construction of expression vector for each subunit of aCEA-IL12. G3, $(GGGGS)_3$.

ious biological activities and may prove to be useful in immunotherapy using T-lymphokine activated killer (LAK) cells.

Materials and methods

Vector construction of IL-12 and aCEA-IL12

IL-12. The gene encoding p35 or p40 was inserted into a T7 promoter-based expression vector, pUT, [29] by a PCR method, as follows. Each subunit was amplified using primers that annealed to the endogenous secretion sequences and included restriction enzyme sites *Nco*I at the 5′ end and *Sac*II at the 3′ end. Restriction enzyme-digested PCR products were cloned into the *Nco*I and *Sac*II sites of the expression vector.

aCEA-IL12. On the basis of the crystal structure of IL-12 [18], aCEA-IL12 was designed as follows: the C-terminus of the p40 domain of human IL-12 was linked to the N-terminus of the T84.66 VL region, and the N-terminus of the p35 domain was linked to the C-terminus of the T84.66 VH region (Fig. 1B). aCEA-IL12 was therefore composed of two units, VH-p35 and p40-VL. To construct VH-p35, the p35 gene fragment was inserted into the 3′ end of the T84.66 IgG VH gene. To make p40-VL, the p40 gene fragment was inserted into the 5′ end of the T84.66 IgG VL gene. pRA vector [28,30,33] was used as an expression vector. The tandem restriction enzyme sites *Nco*I–*Eag*I-$(GGGGS)_3$ linker-*Eco*RV–*Sac*II (Fig. 1B) were used for insertion of the genes into the expression vector.

Expression and purification of each polypeptide chain

Expression and purification of each polypeptide chain were performed according to the method of Tsumoto et al. [29]. In brief, *E. coli* strain BL21 (DE3) transformed with expression vector was incubated at 37 °C in 2× YT broth. When the optical density had reached 0.8 at a wavelength of 600 nm, 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to the culture to induce protein production and the cells were grown overnight. Cell pellets separated from 200 mL culture by centrifugation (2000*g*, 35 min) were resuspended in 10 mL of 20 mM Tris–HCl (pH 8.0 at 20 °C), ultrasonicated at 150 W for 15 min, and centrifuged at 4500*g* for 20 min. Then the intracellular insoluble (ICIS) fraction was solubilized with 10 mL of 6 M guanidium hydrochloride (GdnHCl)–Tris–HCl (pH 8.0 at 20 °C) overnight at room temperature. After solubilization, the proteins were purified by immobilized metal affinity chromatography (IMAC) using a Talon metal affinity resin column (Clontech).

Refolding of IL-12 and aCEA-IL12 by AISD

AISD was applied to the refolding to obtain functional IL-12 and aCEA-IL12 from the ICIS fraction. Each purified subunit of recombinant IL-12 (rIL-12) and aCEA-IL12 was diluted to 7.5 μM with 6 M GdnHCl–50 mM Tris–HCl (pH 8.0 at 4 °C) and mixed with the same molar ratio of each subunit. AISD was then used as reported previously [29,30,33]. To enhance the refolding efficiency we introduced shuffling of disulfide bonds, i.e., 3.75 mM of reducing reagent (glutathione, reduced form; Sigma) and 375 μM of oxidizing reagent (glutathione, oxidized form; Sigma) were added to the refolding solution when the concentration of GdnHCl was below 1 M.

Gel filtration

Gel filtration was used to quantify purified refolded rIL-12 and aCEA-IL12. Samples were chromatographed in an FPLC system with a Superdex 200 26/60 column (Amersham Biosciences) operated at 3 mL/min at 4 °C under 50 mM Tris–HCl (pH 8.0 at 4 °C) containing 200 mM NaCl as a running buffer. Re-chromatography of aCEA-IL12 was performed with a Superdex 200 10/30 column (Amersham Biosciences) operated at 0.5 mL/min at 4 °C under the same buffer system described above.

SDS–PAGE and Western blotting

Five to ten micrograms of proteins in each fraction, precipitated with 6% trichloroacetic acid and 0.083% deoxycholate, was subjected to 0.1% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions, and then stained with Coomassie brilliant blue R-250. The proteins were blotted onto nitrocellulose membranes (Amersham Biosciences) and incubated with peroxidase-conjugated anti-His6-tagged monoclonal antibody (mAb) (Invitrogen). This was followed by signal enhancement with an ECL Detection System (Amersham Biosciences).

Flow cytometric analyses

The C-terminal c-myc tag attached to each subunit of aCEA-IL12 was used for flow cytometric analyses. Test cells (5×10^5) were first incubated on ice with 10 μg aCEA-IL12 or with 10 μg of the parental antibody, T84.66, for 30 min. After being washed in phosphate-buffered saline (PBS) containing 0.1% NaN_3, the cells were exposed to the FITC-conjugated anti-c-myc mAb 9E10 (Santa Cruz Biotechnology) as the fluorescence labeling Ab. The stained cells were analyzed by flow cytometry (FACS Calibur, Becton–Dickinson).

Proliferation assay of PHA-activated PBMCs

Preparation of phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs). PBMCs isolated by density-gradient centrifugation from a healthy volunteer were cultured in medium containing 0.1% PHA (Gibco). After three days, recombinant interleukin 2 (rIL-2), kindly provided by Shionogi Pharmaceutical (Osaka, Japan), was added to the culture to give a final concentration of 50 IU/mL. Then an additional incubation was performed for 24 h.

Proliferation assay. Proliferation of PHA-activated PBMCs was assessed by a cell proliferation by an enzyme-linked immunosorbent assay (ELISA) system (Amersham Biosciences). PHA-activated PBMCs (1×10^5) suspended in 0.2 mL of culture medium were distributed into each well of 96-well flat-bottomed plates (Sumitomo Bakelite, Osaka, Japan) in the presence of various concentrations of the prepared proteins. After incubation of the plates for 48 h at 37 °C, 5-bromo-2′-deoxyuridine (BrdU) labeling reagent was added and the cells were incubated for a further 24 h. The detection procedure was performed in accordance with the manufacturer's assay protocol for cells in suspension. Optical density was measured with a plate reader (Bio-Rad, model 3550) at a wavelength of 450 nm.

LAK induction assay

Human IL-12 synergizes with a low concentration of rIL-2 to induce LAK cell activity [19]. PBMCs were incubated in 24-well tissue culture plates, each containing 1 mL culture (7.5×10^5 cells/well). Some cultures received human IL-2 at a concentration of 5 IU/mL and/or the refolded protein was added in various concentrations. All cultures were incubated for three days at 37 °C. The cytotoxic activity of these cells was assayed by 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS)-based cytotoxic assay (see the section below on in vitro growth inhibition assay).

In vitro growth inhibition assay

The production of T-LAK cells was followed as described previously [30,33]. In vitro growth inhibition assays of various cell lines were performed with an MTS assay kit (CellTiter 96 AQueous Non-radioactive Cell Proliferation Assay; Promega Madison, WI). Target cells (5000 cells in 100 μL culture medium) were distributed to each well of a half-area (A/2) 96-well flat-bottomed plate (Costar, Cambridge, MA). The cells were cultured overnight to enable them to adhere to the bottoms of the wells. After the culture medium had been removed by aspiration, 100 μL of T-LAK cells plus various concentrations of each refolded protein was distributed into each well. After culture for 48 h at 37 °C, then each well was washed with PBS three times to remove T-LAK cells and dead target cells. This was followed by addition of 95 μL/well of culture medium and 5 μL/well of a fresh mixture of MTS–phenazine methosulfate solution (Promega). The plates were read on a microplate reader (Bio-Rad, model 3550) at a wavelength of 490 nm after incubation for 1 h at 37 °C. Growth inhibition of target cells was calculated as follows: % growth inhibition of target cells = $[1 - (A_{490}$ of experiment $- A_{490}$ of background$)/(A_{490}$ of control $- A_{490}$ of background$)] \times 100$.

Growth inhibition assay with a sample washing step has been performed as follows. The samples at certain concentrations were added to TFK-1 cells in the wells and incubated for 1 h at room temperature. The supernatant of each well was removed and the cells were washed twice by PBS buffer. T-LAK cells were then added to the wells at an effector to target ratio of 5:1. The following steps were similar to the procedures as described above.

Results

Preparation of refolded IL-12 and aCEA-IL12 by the AISD method

Each polypeptide of rIL-12 and Fv-fused protein (aCEA-IL12) was expressed in *E. coli* BL21 (DE3) harboring the plasmid constructed for expression of each subunit. SDS–PAGE and Western blotting using anti-His_6-tagged Ab showed that each of rIL-12 and aCEA-IL12 subunits was present mainly in the intracellular insoluble fraction (Fig. 2). The yield of each polypeptide chain from this fraction was more than 10 mg/1 L of culture.

To obtain soluble rIL-12 and aCEA-IL12 from the inclusion bodies, we optimized the refolding system of that we used in our previous study [29,33]. First, the insoluble fraction was solubilized and dissociated in 50 mM Tris–HCl (pH 8.0 at 4 °C) containing 6 M GdnHCl and 200 mM NaCl, and the proteins were purified by IMAC as described in Materials and methods. Each highly purified polypeptide was confirmed by SDS–PAGE analysis to exist as a single band (Fig. 2, lanes 3 and 6). The purified proteins were diluted with elution buffer to 7.5 μM and refolded by AISD after mixing at equal molar ratios of each subunit, as described above. During refolding no aggregation has been observed.

Gel filtration analyses of refolded rIL12 solubilized by AISD showed multimeric soluble aggregates of p35 and p40, heterodimer, and monomer of p40 (Figs. 3A(a) and B(a)). Up to 10% of solubilized protein was refolded as heterodimer; in contrast, less than 1% of the refolded protein is heterodimer if refolding procedures such as conventional dilution methods and stepwise dialysis method without additives are used (data not shown). On the other hand, gel filtration analyses of aCEA-IL12 solubilized via AISD showed mainly multimeric soluble aggregates and heterodimer (Fig. 3A(b)). SDS–PAGE and anti-His tag Western blotting analyses of heterodimer fraction of refolded aCEA-IL12 indicated that p40-VL and VH-p35 form heterodimer via disulfide bonds (Fig. 3B(b)). More than 40% of solubilized protein was refolded as heterodimer. Re-chromatography of this fraction shows monodisperse single peak (Fig. 3A(c)). These results suggest that fusion of an antibody variable domain markedly induced heterodimerization. The heterodimeric fractions of refolded rIL-12 and aCEA-IL12 were subjected to biological assay as described below.

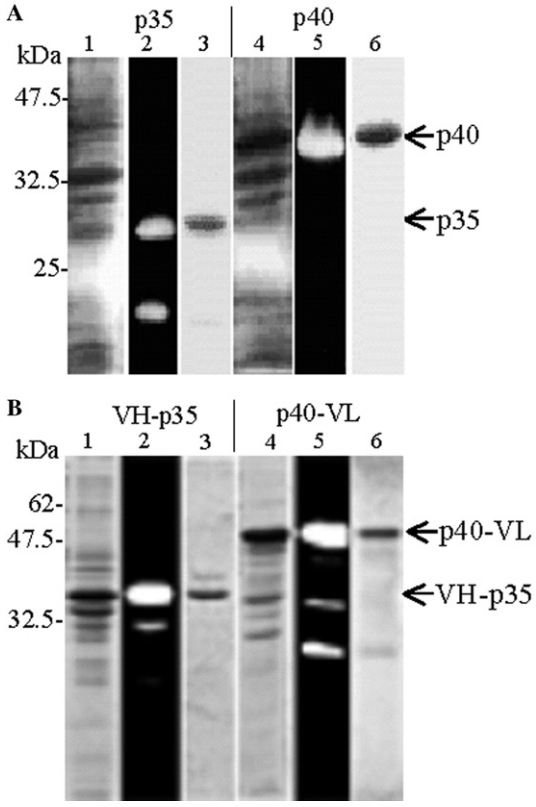

Fig. 2. Expression and purification of rIL-12 and aCEA-IL12 SDS–PAGE under reducing conditions and Western blot analyses by anti-His6-tag mAb of fractions of the inclusion body from *E. coli* BL21 (DE3) cells expressing rIL-12 (A) and aCEA-IL12 (B) and the fractions eluted from immobilized metal affinity chromatography under denaturing conditions. Lanes 1, 4: SDS–PAGE of bacterial intracellular insoluble fraction. Lanes 2, 5: Western blotting analyses of lanes 1 and 4, respectively, using anti-His tag mAb. Lanes 3, 6: SDS–PAGE of the fractions eluted from immobilized metal affinity chromatography.

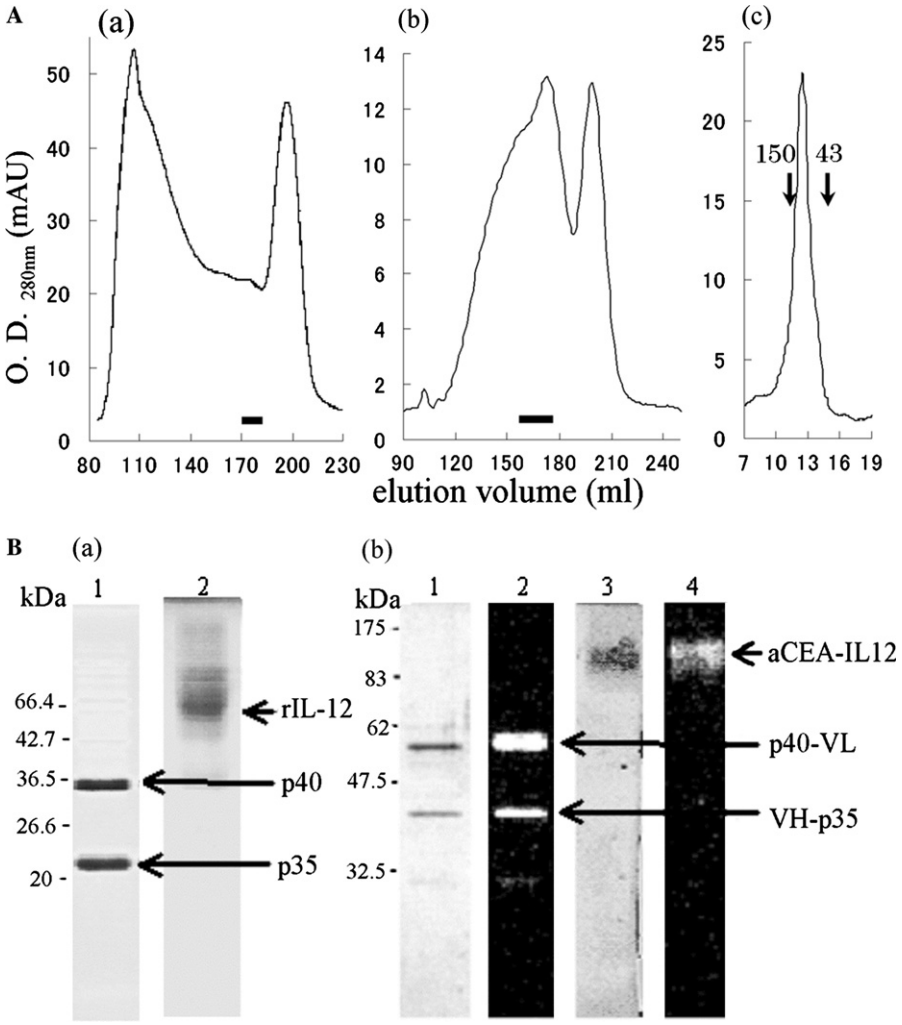

Fig. 3. Gel filtration and SDS–PAGE of refolded proteins. (A) Profiles of gel filtration. (a) rIL-12, (b) aCEA-IL12. Collected fractions used for biological assay were indicated as solid lines at the bottom of the profiles; (c) re-chromatogram of aCEA-IL12 using gel filtration on Superdex 200. Molecular weight markers are indicated with solid arrows. (B) SDS–PAGE of the refolded proteins. (a) rIL-12. Lanes 1 and 2, under reducing and non-reducing condition, respectively; (b) aCEA-IL12. Lanes 1 and 3, under reducing and non-reducing condition, respectively; lanes 2 and 4, Western blotting of aCEA-IL12 using anti His-tag mAb under reducing, and non-reducing condition, respectively.

Bioactivity of refolded rIL-12 and aCEA-IL12

LAK cell induction assay and IFN-γ production assay

The biological activity of refolded rIL-12 or aCEA-IL12 was estimated by LAK induction assay and IFN-γ production assay. PBMCs cultured with the refolded protein synergized with a low concentration of human rIL-2 (5 IU/mL) showed cytotoxic activity, whereas the refolded protein alone did not induce LAK cells (data not shown). Specific production of IFN-γ in response to addition of refolded rIL-12 of aCEA-IL12 was also observed (data not shown).

PHA-activated PBMC proliferation assay

The potential of refolded rIL-12 to stimulate the proliferation of PHA-activated PBMCs was examined by BrdU incorporation followed by ELISA. We observed effective stimulation of proliferation of PHA-activated PBMCs, which was dependent upon the concentration of refolded rIL-12 from 1 pM to 1 nM (Fig. 4). There was no remarkable difference in the concentration of rIL-12 over the range. These results showed that refolded rIL-12 had a marked effect on the proliferation of PHA-activated PBMCs. We also estimated the bioactivity of aCEA-IL12. aCEA-IL12 induced the proliferation of PHA-activated PBMCs at a concentration of 0.5 nM (Fig. 4), indicating that aCEA-IL12 had similar biological activity to refolded rIL-12.

Binding of aCEA-IL12, as studied by flow cytometry

We assessed the binding activity of aCEA-IL12 for CEA antigen by flow cytometry using CEA-positive or negative cell lines (Fig. 5). Refolded aCEA-IL12 could bind to CEA-positive TFK-1 (human bile duct

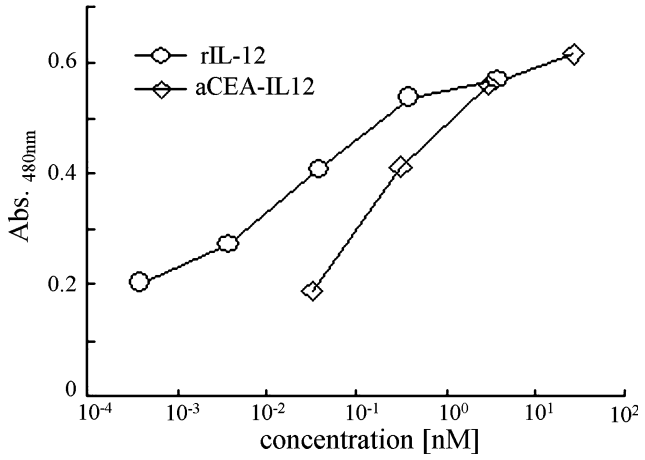

Fig. 4. Proliferation assay of PHA-activated PBMCs (BrdU incorporation test). Freshly isolated PHA-activated PBMCs were incubated for 72 h with the indicated doses of refolded rIL-12 (circles) and aCEA-IL12 (rhombi). BrdU was then added to the culture. Incorporated BrdU was measured by the cell proliferation ELISA system (see details in Materials and methods).

carcinoma), Sq-19 (human squamous cell carcinoma), and OBA-LK1 (human lung carcinoma) cells but could not bind to CHO-K1 (Chinese hamster ovary cell; CEA-negative). The results were almost identical to those for the parental anti-CEA antibody T84.66 IgG (Fig. 5). Moreover, all recombinant antibodies inhibited the binding of parental T84.66 IgG to both TFK-1 and OBA-LK-1 cells (data not shown), indicating that aCEA-IL12 recognized the same epitope. From these results, we can conclude that aCEA-IL12 has binding activity to CEA-positive cell lines and may have the potential to facilitate the activation of immune cells at tumor sites.

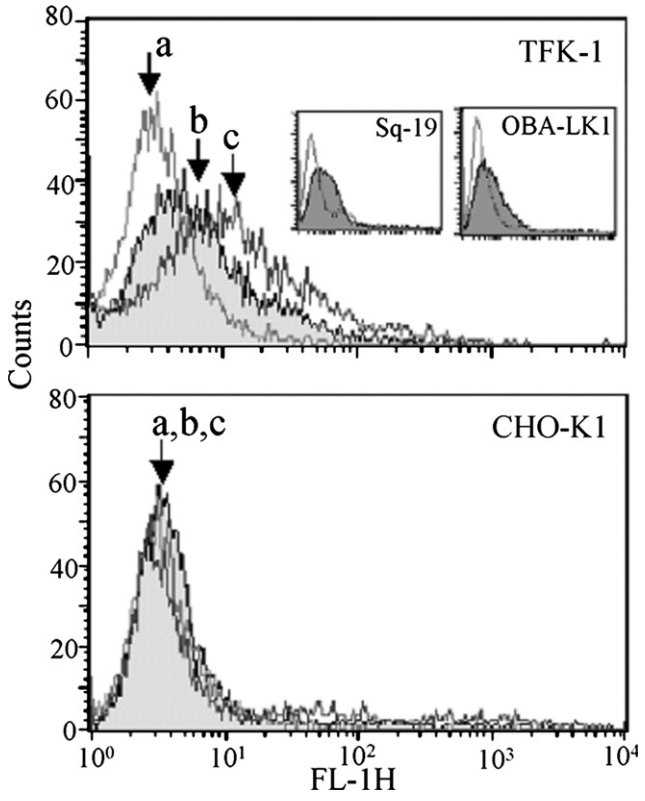

Fig. 5. Flow cytometric analyses of aCEA-IL12 for CEA-positive cancer cell line (TFK-1) or CEA-negative one (CHO-K1). Cells were incubated without (a) or with (b) aCEA-IL12 and stained with FITC-conjugated mouse anti-c-myc mAb 9E10; (c) black solid line, cells incubated with T84.66 IgG and stained with FITC-conjugated rabbit anti-mouse IgG antibody. The results for other CEA-positive cancer cell lines (Sq-19 and OBA-LK1) are shown in the inset.

Mediation of the effect of T-LAK cells on growth inhibition of TFK-1 cells

We evaluated the anti-tumor-enhancing effects of refolded rIL-12 and aCEA-IL12 in a growth inhibition assay using human bile duct carcinoma TFK-1 cells as the target and T-LAK cells as the effector. Strong enhancement of the effect of T-LAK by aCEA-IL12 was confirmed at an effector-to-target (E:T) ratio of 5:1 (Fig. 6). The effect of aCEA-IL12 in inhibiting the growth of TFK-1 was found to be saturated up to 100 nM.

We then analyzed the binding activity of aCEA-IL12 to cancer cells by growth inhibition assay with a washing step. The cancer cells were incubated with the refolded samples, washed, and then cultured with T-LAK cells at an E:T ratio of 5:1. The growth inhibition of TFK-1 cells was observed only when aCEA-IL12 was added at a concentration of above 20 nM (Fig. 7); on the other hand, the refolded rIL-12 and T84.66 IgG had no effect on the tumor growth (Fig. 7).

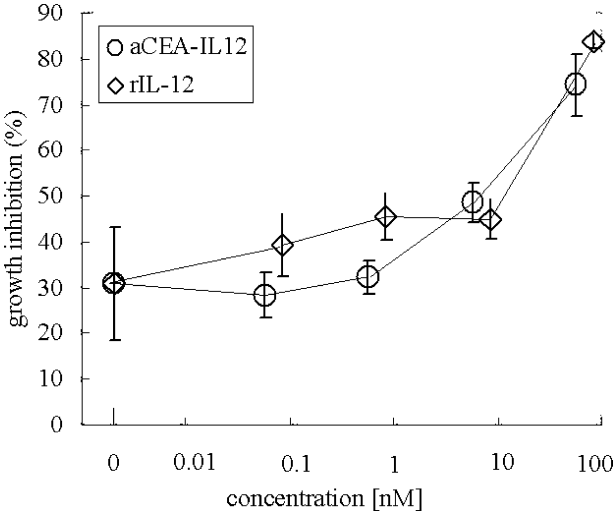

Fig. 6. Growth inhibition assay of the human bile duct carcinoma line TFK-1. Growth inhibition indices were determined by a 48-h MTS assay, in which the sample (refolded rIL-12 or aCEA-IL12) and T-LAK cells (effectors) were added to TFK-1 cells (targets) at an effector to target ratio of 5:1. Data are mean values from at least three independent tests.

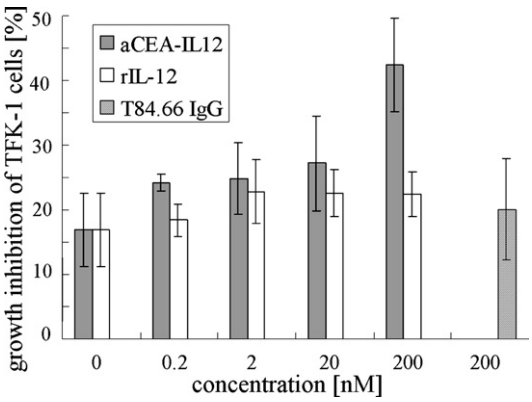

Fig. 7. Growth inhibition assay with a sample washing step. The samples at certain concentrations were added to TFK-1 cells in the wells and incubated for 1 h at room temperature. The supernatant of each well was removed and the cells were washed twice by PBS buffer. T-LAK cells were then added to the wells at an effector to target ratio of 5:1. The following steps were similar to the procedures as described in Materials and methods. Data are mean values from at least three independent tests.

Discussion

Here we report an efficient system of refolding human rIL-12 and its fusion protein aCEA-IL12 as a heterodimer by using AISD. We successfully prepared disulfide-locked heterodimeric protein using AISD, despite a relatively low yield, i.e., up to 20% of total proteins refolded. These results show that introduction of disulfide-shuffling conditions at particular stages of GdnHCl concentration in AISD can markedly increase the amount of product correctly refolded.

The heterodimeric fraction of the refolded samples showed the specific biological activity of human IL-12, which was identical to mammalian cell CHO-expressed recombinant human IL-12. Subunits of human IL-12 were also refolded separately and mixed with an equal molar ratio of each subunit under disulfide shuffling conditions. Subsequent assay showed that the mixture of independently refolded p35 and p40 showed no biological activity (data not shown). Surface plasmon resonance analysis of the interaction between refolded p35 and immobilized refolded p40 did not show a specific interaction (data not shown). These results clearly indicate the importance of introducing an equal molar ratio of both subunits into the solution to be refolded. Therefore, cooperative refolding of each subunit, including intermolecular disulfide bond formation, may be particularly important for preparation of functional refolded IL-12. It should be noted that refolding of one chain of antibody variable domain reduces the refolding of another chain: both chains have been suggested to refold cooperatively [30,37].

We constructed tumor-specific IL-12 protein, i.e., IL-12 fused to an antibody variable domain, designated as aCEA-IL12. The heterodimeric fraction of refolded aCEA-IL12 showed both the biological activity of IL-12 and the specific binding property of anti-CEA antibody, indicating that each domain of the fusion protein was refolded properly and, therefore, bifunctional. Growth inhibition assay showed that enhancement of the killer activity of T-LAK toward a CEA-positive cell line was dependent upon the concentration of the protein. The refolding yield of aCEA-IL12 was improved in comparison with that of refolded IL-12, i.e., up to 50% of total proteins were refolded. The antibody Fv region may induce heterodimer formation and may also reduce the incidence of wrong intermolecular interaction during refolding and suppress aggregate formation. It is known that the p40 subunit forms a homodimer and has an antagonist activity [38]. Recent reports have shown that the IL-12 multimer is the major product in the case of recombinant expression systems [30]. Our results indicate that the Fv fragment can effectively function as an adapter of heterodimer formation, and, therefore, formation of the p40 homodimer can be effectively avoided by fusion with the Fv fragment.

Finally, we must emphasize that the construction of fusion proteins using antibody variable domains, as reported here (aCEA-IL12), may be applicable to various heterodimeric proteins. Coiled–coil association (e.g., leucine zipper) has been used for oligomerization of proteins [39,40]. Here, we showed that antibody variable domains can also facilitate association of each subunit of a heterodimer. Appropriate construction of fusion proteins using antibody variable domains may be a powerful tool, not only for stabilization of the heterodimer but also for grafting bifunctional properties. Immune-activating cytokines and their antibody fusion proteins may nowadays be powerful tools in cancer therapy, and use of the refolding system reported here may facilitate the large-scale preparation of protein drugs.

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