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Anti-tumor activity and immunogenicity of a mutated staphylococcal enterotoxin C2

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Received October 9, 2012, accepted November 9, 2012

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Pharmazie 68: 359–364 (2013) *doi: 10.1691/ph.2013.2824*

In this study, a novel SEC2 mutant with lower toxic activity, named 2M-118 (H118A/T20L/G22E), was engineered by site-directed mutagenesis of structural domains that are responsible for MHC class II molecule binding and TCR binding, respectively. Stimulating activity on murine splenocytes, anti-tumor effect and immunogenicity of 2M-118 were investigated in BALB/c mice. 2M-118 not only remained splenocyte stimulation activity, but also effectively inhibited the growth of S180 sarcoma in the BALB/c mice. Even though antibodies to 2M-118 could be induced after repeated administration, the action of 2M-118 was hardly neutralized or cross neutralized. Like other superantigens, immunosuppression could happen when 2M-118 was given at a greater dose. In conclusion, 2M-118 is a promising anti-tumor drug candidate for its acceptable toxicity and satisfying anti-tumour efficacy.

1. Introduction

Staphylococcal enterotoxins (SEs) are exotoxins secreted by *Staphylococcus aureus* (Bohach et al. 1990), and can be classified into many major serological types, including SEA to SEE, SEG to SET, and SEU (Dinges et al. 2000; Hovde et al. 1990; Letertre et al. 2003; Munson et al. 1998; Ono et al. 2008; Su Wong 1995). SEC can be further classified into three subtypes (C1, C2 and C3) by minor differentiation at 10 residues in amino acid sequence (Hovde et al. 1990). As superantigens, SEs can stimulate polyclonal T-cell proliferation, leading to release of large amounts of various cytokines such as IL-2 and IFN-γ (Carlsson Sjogren 1985), by binding to the outside of the antigenic groove of major histocompatibility complex class II (MHC class II) molecules and V_β regions of T cell receptor (TCR) (Dinges et al. 2000). It is based on direct cell-mediated cytotoxicity and the action of various antineoplastic cytokines that SEs might serve as a kind of promising drugs for systemic antitumor immunotherapy. However, severe emetic and pyrogenic effects, and even fatal shock are so common for these superantigens that their clinical applications are limited.

To find a potential antitumor SE, relations of structure and activity of some SEs have been investigated, and great achievements have been reached (Fraser et al. 1992; Hoffman et al. 1996; Li Y. F. et al. 2004; Li Z. J. et al. 2009; McCormick et al. 2003; Ono et al. 2008; Papageorgiou et al. 2004; Pless et al. 2005; Saline et al. 2010). As is known, histidine residues at Zn-binding sites of SEA usually play an important role in exerting bioactivity, and His225 of SEA, for example, is responsible for both superantigen and emetic activities, whereas His61 appears to be the cause for emetic activity (Hoffman et al. 1996). Harris also confirmed that the emetic activity and T cell proliferation stimulating activity can be dissociated (Harris et al. 1993). Therefore, a predominant strategy in the development of such kinds of drugs is to find and modify the activity-related domains of the structures of SEs to produce mutated proteins with greater antitumor efficacy as well as lower harmful potency (Kumaran et al. 2001; Papageorgiou et al. 1995, 2004; Schad et al. 1995; Swaminathan et al. 1992). These findings suggest that T cell stimulation and toxic activities are separable in SEs and thus modification of SEs by site-directed mutagenesis is a good way to find a satisfactory agent for anti-tumor immunotherapy.

Crystal structure of SEC2 showed that histidine residues at positions 47, 118 and 122 are necessary for the formation of the two Zn-binding domains. The mutation of the residue at position47 may impair the stimulating activity of SEC2 for T lymphocyte proliferation. Histidines at positions 118 and 122 are responsible for emetic and febrile actions of SEC2, and the substitution of histidine at position 118 or 122 has introduced a decrease in such adverse effects without any impairment of superantigen activity (Papageorgiou et al. 1995, 2004; Wang et al. 2009). Other studies indicated that the residues at positions 20 and 22 play an important role in TCR binding and multiclonal T lymphocyte activation (Lamphear et al. 1998; Papageorgiou et al. 1995; Schad et al. 1997). To get recombinant protein mutants with greater antitumor activity as well as less toxicity, a novel mutated SEC2, 2M-118(H118A/T20L/G22E), was designed and purified, in which histidine at position 118 is replaced with alanine, threonine at position 20 replaced with leucine and glycine at position 22 replaced with glutamine. Our previous studies suggested that among tens of the mutants, 2M-118 exhibited potent *in vitro* antitumor activity and least emetic toxicity (Data not shown here). In addition, as a kind of superantigens, the enterotoxins could inactivate or delete their reactive T cell subsets, especially when used for long term or with a large dose (Rellahan et al. 1990; Weber et al. 2000; White et al. 1989). Therefore,

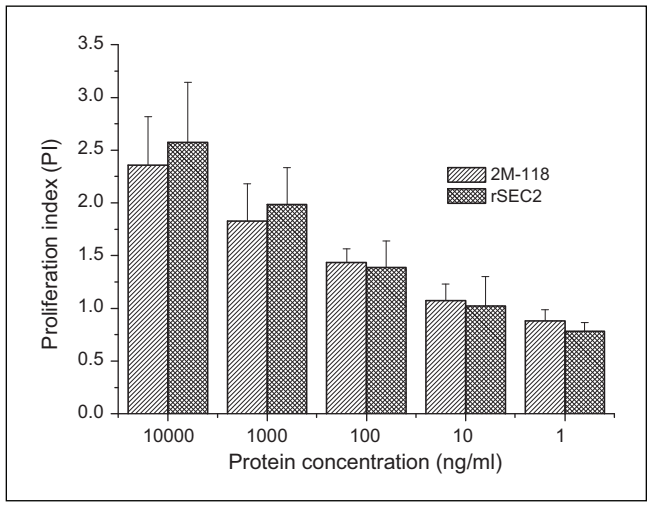

Fig. 1: Proliferative effects of 2M-118 and rSEC2 on murine spleen lymphocytes. Various levels of 2M-118 or rSEC2 were incubated with 5×10^6 cells/mL murine spleen lymphocytes (final concentration) at 37 °C in 5% CO_2 atmosphere for 48 h, and then cell proliferation was measured by an MTT method. The degree of lymphocyte proliferation was expressed as "PI ± SD" (mean values, with bars indicating SD). Each level of 2M-118 or rSEC2 was tested four times

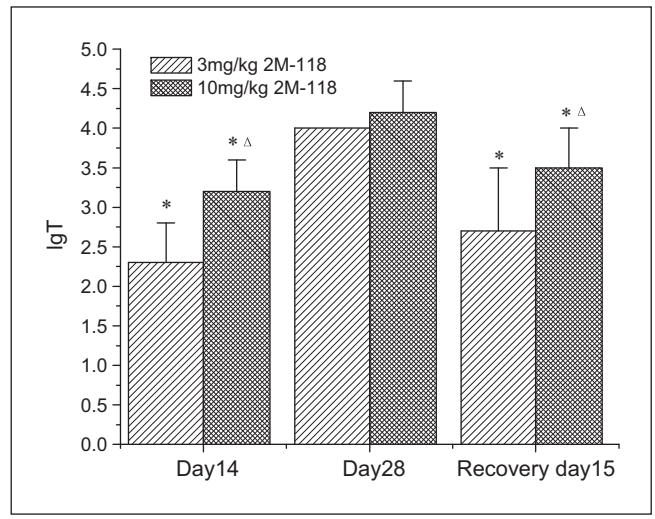

Fig. 2: Antibody titers of the sera of the mice receiving 2M-118 on days 14, 28, and recovery day 15 after the last administration of 2M-118 The titer of anti-2M-118 was determined by an indirect ELISA method. The titer was dose-dependent, and the maximal value appeared on day 28. An asterisk means that the titers for a dose on day 14 and on recovery day 15 are significantly different from that for the identical dose on day 28, respectively ($P < 0.05$). A delta indicates that there is significant difference in the titers for 10 mg/kg 2M-118 on day 14 and on recovery day 15, compared with 3 mg/kg 2M-118 ($P < 0.05$)

in this study, the activity of 2M-118 in stimulating T lymphocyte proliferation and *in vivo* anti-tumor effects of 2M-118 at greater doses in BALB/c mice were investigated.

As is well known, therapeutic proteins, when given to the receivers, are generally immunogenic, which could lower the efficacy of some protein drugs, or introduce untoward effects such as serum sickness like disease, infusion reactions, anaphylaxis and anaphylactoid reactions (Cook et al. 2007; de Vries et al. 2009; Schellekens 2002; 2005; Wolbink et al. 2009). It is necessary for almost all therapeutic proteins to investigate the immunogenicity in their preclinical studies. In this study, immunogenicity property of 2M-118 was preliminarily evaluated.

2. Investigations and results

2.1. Expression and purification of proteins

The expression vectors for rSEC2 and 2M-118 without His-tag were constructed and confirmed by DNA sequencing. Proteins were induced by IPTG at 30 °C for 4 h, and soluble expression parts were purified with SP Sepharose FF cation exchange column and Q Sepharose FF anion exchange, successively. The purities of both rSEC2 and 2M-118 were over 98% (SDS-PAGE visualized by Coomassie blue staining).

2.2. Murine spleen lymphocyte proliferation assay

The ability of 2M-118 to stimulate murine T-cell proliferation was tested. The results showed that 2M-118 and rSEC2 exhibited a similar concentration–dependent stimulating effect on the proliferation of murine spleen lymphocytes within a range of 1 ng/mL and 10000 ng/mL, and no statistically significant difference in stimulating potency (expressed as "proliferation index(PI)") was seen between the two proteins ($P > 0.05$) (Fig. 1).

2.3. Anti-tumor activity of 2M-118 in vivo

In order to study the antitumor activity of 2M-118, the BALB/c mouse tumor model xenografted with S180 sarcoma was applied. The results showed that the tumor inhibition rates (TIR) in 2M-118-treated group dose-dependently increased from 45.82% to 60.95% when 2M-118 was given intravenously at doses from 5 mg/kg to 10 mg/kg. However, when the dose went up to 20 mg/kg, the TIR reduced to 41.15% rather than increased with the dose. This might be attributed to immunosuppression (Banz et al. 2002; Cauley et al. 2000).

For the survival of the mice, 11 out of 14 mice survived with the dose of 10 mg/kg, and nearly half of the mice died when given 20 mg/kg, which suggested that 2M-118 has potent toxicity when used in high doses. Fortunately, the superantigen is commonly applied at very low doses, far below its immunosuppressive or lethal doses (Table 1).

2.4. Immunogenicity test

An indirect ELISA method was used for the determination of the titer of the antibodies to 2M-118. The antibodies to 2M-118 were detected in all the three-time-pointed serum samples, and the antibody titer on day 28 was the highest. The antibody titer on recovery day 15 went down to that on day 14 (Fig. 2). Statistical analysis indicated that the antibody titers were significantly different for the samples tested ($P < 0.05$), and the antibody titers for 10 mg/kg 2M-118 was greater than that for 3 mg/kg, with the number of the antibody-positive mice going up with the increase of the dosage (Table 2).

In the test for neutralizing activity of the anti-2M-118, compared with PI for 2M-118 treated with negative control, PI of 2M-118 was reduced by 20% by the pooled anti-sera. Even though there was significant difference in PI between the pooled anti-sera of anti-2M-118 and anti-rSEC2 ($P < 0.05$), most stimulating action of 2M-118 on splenocytes was retained. In contrast, for rSEC2, PI was drastically lowered by the pooled anti-sera, and significant difference in PI existed between the negative control and the pooled anti-sera ($P < 0.05$) Fig. 3.

3. Discussion

Staphylococcal enterotoxin C2 has been applied as a predominant active ingredient to antitumor therapy in China for many years. As one of the superantigens, SEC2 exerts its superantigen-dependent cell mediated cytotoxicity (SDCC) effects on tumor

Table 1: Tumor growth inhibition rate of 2M-118 (Tumor weights shown as "mean ± SD")

Groups	Dose (mg/kg)	Tumor weight (g)	Inhibition rate (%)	Ratio (live/total)
Normal saline	—	2.187 ± 0.585	—	19/19
Cyclophosphamide	50(i.p.)	0.630 ± 0.232[a]	71.19	19/20
Low dose group	5(i.v.)	1.185 ± 0.368[a,b]	45.82	12/12
Mid dose group	10(i.v.)	0.854 ± 0.357[a,b]	60.95	11/14
High dose group	20(i.v.)	1.287 ± 0.470[a,b]	41.15	6/10

[a] the tumor weight is significantly different from that of normal saline group ($P < 0.01$). [b], The tumor weight is significantly different from that of cyclophosphamide group ($P < 0.05$).

Table 2: Antibody titer to 2M-118 with different doses at three sampling time points (logarithm of titer ± SD)

Groups	Dose (mg/kg)	lg T[a]		
		Day 14	Day 28	Recovery day 15
Normal saline	—	ND	ND	ND
Low dose group	3	2.3 ± 0.5[c] (9/10)	4.0 ± 0.0 (9/10)	2.7 ± 0.8[c] (7/10)
High dose group	10	3.2 ± 0.4[b,c] (10/10)	4.2 ± 0.4 (10/10)	3.5 ± 0.5[b,c] (8/10)

[a] T means antibody titer; [b], Compared with that for 3 mg/kg 2M-118, lgT for 10 mg/kg 2M-118 is significantly greater on the identical day ($P < 0.05$). [c], For 10 mg/kg 2M-118, lgTs on day 14 and on recovery day 15 are statistically different from that on day 28 ($P < 0.05$). The ratio of the number of the antibody-positive mice to the number of the mice in the group is shown in the bracket. ND means that no antibody was detected in the normal saline group.

cells only by binding initially to the outside of the antigenic groove of major histocompatibility complex class II (MHC II) molecules and then being presented to T cells and binding to the TCRs with Vβ chain.

However, non-selective binding to the MHC class II molecule-positive non-cancer cells will introduce severe toxicity effects like fever, vomiting and shock, which limits the clinical applications of SEC2 (Erlandsson et al. 2003; Holzer et al. 1997). Besides, high affinity for MHC class II molecules also leads to antibodies against SEC2, which might lead to treatment failure. In a previous study with SEA and SEE, antibody binding sites were found around the MHC class II binding sites using epitope mapping. When these epitopes were genetically removed, both the toxicity and the immunogenicity of the mutated SEA were reduced greatly (Erlandsson et al. 2003).

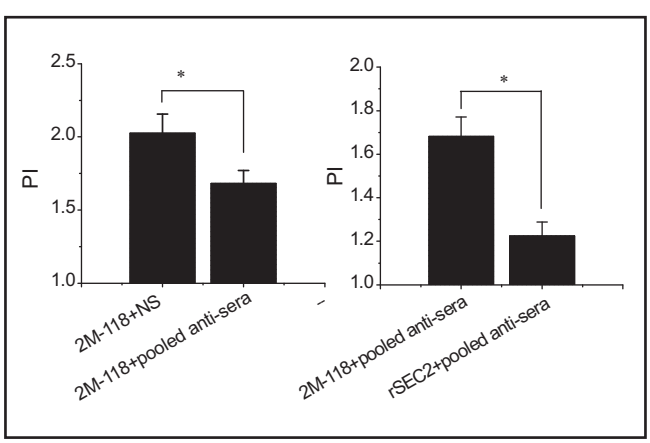

Fig. 3: Neutralizing activity of the pooled anti-sera against 2M-118 and rSEC2. 2M-118 or rSEC2 was mixed with the 10-fold diluted pooled sera from the mice intravenously receiving 3 mg/kg rSEC2 or 2M-118 twice a week for four weeks, respectively. Sera from the mice receiving normal saline were used as negative control. After a two-hour binding of the antibodies to 2M-118 or rSEC2, mouse spleen cells(1×10^7 cells/mL) was added to and incubated at 37 °C in 5% CO_2 for 72 h. Proliferation of murine splenocytes (PI) was measured at 490 nm with an MTS method. Data are shown as "means of triplicates ± SD". An asterisk indicates that PIs are significantly different between two groups ($P < 0.05$). Right, comparison of PI values of 2M-118 and rSEC2 treated with the pooled anti-sera. Left, comparison of PI values of 2M-118 treated with the pooled anti-sera and with the sera from the mice receiving normal saline

The former studies have shown that engineered SEs by site-directed mutagenesis could retain their superantigenicity while reducing their toxicity. A successful example was about a mutated SEA, which kept its capability of stimulating T lymphocyte proliferation at low doses without emetic effects (Hoffman et al. 1996). The mutated SEA(SEA(D227A)) with lower affinity with MHC class II molecules was less neutralized by anti-serum and activated T lymphocytes as effectively as its wild counterpart. The fusion protein from Fab of a monoclonal antibody and SEA(D227A) was successfully targeted to the MHC class II molecule negative tumor cells by superantigen antibody dependent cell-mediated cytotoxicity (SADCC), and entered into the phase II clinical trial (Forsberg et al. 2001; Shaw et al. 2007). For SEC2, it is now clear that Asp83, His118, His122 and Asp9 from the neighbouring molecule constitute one zinc binding site of SEC2. His47 and Gln71, and Gln119 and Gln80 from the neighbouring molecule form the other zinc ion binding domain. The former domain is responsible for binding to MHC class II molecules and associated with the toxic activity whereas the latter relates to the superantigenic activity of SEC2 (Papageorgiou et al. 1995, 2004). Wang substituted alanine for histidine at position 118 by site-directed mutagenesis and found that the toxicity of the mutated SEC2 was reduced greatly while its superantigenic activity was slightly impared (Wang et al. 2009). That is to say, even for MHC class II molecule-dependent superantigens, such as SEC2, it is possible to lower toxicity and to keep superantigenicity via site-directed mutagenesis strategy. In other studies, the results indicated that the amino acids at positions 20 and 22 of SEC2 reside in putative $TCRV_\beta$-chain binding domain, and these residues play an important role for SEC2 to interact with TCR (Papageorgiou et al. 1995; Schad et al. 1997). The mutated SEC2 (T20L/G22E) exhibited a significantly enhanced superantigen activity and antitumor response, compared with native SEC2 *in vitro* (Wang et al. 2009). Therefore, we constructed here a mutated SEC2, which is named by 2M-118, by simultaneous mutation at residues 118, 20 and 22, and reasonably expected to introduce a novel mutant with both a decreased toxic effect and at least an uncompromised antitumor activity.

In our previous study, we found that compared with rSEC2, the emetic and febrile activity of 2M-118 is greatly reduced (data not shown). Therefore, in this study, the effect of 2M-118 on T lymphocytes was tested. The results showed that 2M-118 was

able to effectively stimulate the proliferation of murine splenocytes, which was similar to rSEC2 within the range from 10 ng/mL to 10,000 ng/mL. The results suggested that the mutation at positions 20 and 22 would offset the impaired effect on T cell stimulation caused by substitution of the residue at the position 118. The results in the present study and others confirmed that the dependence of SEs on MHC class II molecules could be regulated by the interaction of SEs with TCRs of T lymphocytes (Lamphear et al. 1998; Wang et al. 2009; Xu et al. 2011).

We previously found that 2M-118 exhibited an effective *in vivo* anti-tumor activity when given intravenously at 5 mg/kg to BALB/c mice (data not shown). Now, in this study, we attempted to know about what would happen for the anti-tumor effects and safety when the dose of 2M-118 increased. Therefore, a wider dose range from 5 mg/kg to 20 mg/kg was designed to investigate the antitumor activity of 2M-118 in BALB/c mice. The results showed that the weight and size of the tumor nodules were dose-dependently reduced, and tumor growth inhibition rate (TGI) altered from 45.82% to 60.95% when the dose of 2M-118 increased from 5 mg/kg to 10 mg/kg. Nevertheless, the TGI unexpectedly reduced to 41.2% at the dose of 20 mg/kg. The reason for this may be attributable to immunosuppression that frequently happens in superantigen treatment (Jie et al. 2007; Weber et al. 2000). The mechanism behind immunosuppression may be due to activation induced cell death (AICD) by Fas/FasL and cytokine mediation routes (Banz et al. 2002; Cauley et al. 2000). It is believed that the increasing number of CD4 + CD25 + T regulatory cells also plays an important role in down-regulating activated T cells under the condition of high concentrations or prolonged action of SEs (Pontoux et al. 2002; Seo et al. 2007). Therefore, we considered the decrease in TGI of 20 mg/kg 2M-118 to be attributable to the induction of immunosuppression. Furthermore, death of the mice appeared when they received 2M-118 at a dose of 10 mg/kg, which indicated that 2M-118 will introduce toxic effects when administered at larger doses. Fortunately, SEs are a kind of potent cytoimmunity activators that exert desired antitumor effects at ng or pg levels, and no case of immunosuppression has been reported at such low dose level in the clinical applications of Gaojusheng, an injection containing SEC2 as a major active component, in China for nearly 20 years. A conclusion can be drawn that 2M-118 is a both effective and safe antitumor protein when used at lower doses.

It is well accepted that immunogenicity is often observed in therapeutic proteins. That is to say, the frequent side consequence of administering an immunogenic therapeutic protein is the development of neutralizing or non-neutralizing antibodies against therapeutic proteins, which sometimes leads to compromised beneficial efficacy, anaphylaxis and even life-threatening effects (Schellekens 2010). Hence, no or minimal immunogenicity is desired for a therapeutic protein (Bender et al. 2007; Vultaggio et al. 2010). Because little is still known about the immunogenicity feature of 2M-118 as a protein drug candidate, it is necessary and important to study this issue in the preclinical research and development stage. In this study, we examined the levels and neutralizing activity of the antibodies specific to 2M-118 during and after intravenous administration of 2M-118 at doses of 3 mg/kg and 10 mg/kg to BALB/c mice. Antibodies to 2M-118 were detected at the mid-term (day 14) and the end of dosing phase (day 28), and the end of recovery phase (recovery day 15). The highest titer of antibodies occurred on day 28, and the titer of antibodies in the 10 mg/kg 2M-118 group was higher than that in the 3 mg/kg group when measured on day 14. These results suggested that the strength of antibody titer was to some extent proportional to the dose of 2M-118.

In the *in vitro* experiment investigating the neutralizing activity of anti-2M-118 and anti-rSEC2 sera against 2M-118, we found that compared with the negative control, 2M-118 could still effectively stimulate proliferation of splenocytes when treated with both anti-2M-118 and anti-rSEC2, while superantigenic activity of rSEC2 was almost completely disturbed by the pooled anti-sera. This result indicated that 2M-118 was only partially neutralized by anti-2M-118 or cross neutralized by anti-rSEC2, which is superior in immunogenicity to rSEC2. The underlying mechanism for this consequence is not clear. The study on SEA or SEE found that antigenic epitopes centered around MHC class II molecule binding domain, and single substitution of the residues in this area will greatly alter epitopes and hence reduce the immunogenicity property of a mutant protein (Erlandsson et al. 2003; Woody et al. 1997). Considering the differences in amino acid sequences of 2M-118 and rSEC2, we speculate that the substitution of residue^{118} in MHC class II molecule binding domain will be an important impact factor in the alteration of immunogenicity of 2M-118. The actual mechanism for these alterations relies on further studies on the antigenic epitopes of SEC2 and 2M-118.

In this study, we employed BALB/c mice as the animal model to investigate the immunogenicity of 2M-118. Although conventional animal models are poorly immunogenicity-predictive for humanized or recombinant human therapeutic proteins for different antibody responses to such therapeutic proteins between species (Sauerborn et al. 2010), BALB/c mouse is taken as a human related animal model for its similar response in T cell proliferation effects to humans. Besides, 2M-118 is a xenobiotic to both humans and BALB/c, and therefore it can be presumed that the humoral immune responses to 2M-118 in the animal are similar to those in humans and the results of this study in BALB/c mice are to some extent predictive for immunogenicity features of 2M-118 in humans.

In conclusion, a novel SEC2 mutant with lower toxicity was engineered by substituting alanine, threonine and glycine for histidine^{118} leucine^{20} and glutamic acid^{22}, respectively. Similar to rSEC2, 2M-118 exhibited the stimulating effects on murine T cell, and its potent antitumor action exerts within a wide range of concentrations. Additionally, even though antibodies to 2M-118 can be induced after repeated intravenous administration, 2M-118 is basically not neutralized or cross neutralized by induced anti-2M-118 or anti-rSEC2, which means that the biological activity could not be disturbed in patients following long-term treatment with 2M-118. Even for patients who have ever been infected by SEC2-like proteins, cross neutralization may be so slight as not to impair the action of 2M-118.

4. Experimental

4.1. Animals

BALB/c mice (aged 6–8 weeks old, weighing 20–22 g) were obtained from Beijing Veitonglihua Experimental Animal Technology Co. Ltd. (Laboratory animal reproduction license No. SCXK (Jing) 2006–0009, Beijing, China). The animals were maintained on a 12 h light/dark cycle. The animals were raised with open access to standard food and water *ad libitum*. Environmental conditions were maintained at a temperature of 20–25 °C and a relative humidity of 40–70%, 10–15 times per hour ventilation. (Laboratory animal use permit No.: SYXK (Jing) 2006–0002). The animals were allowed to acclimate to the facility for at one week before randomization into the different experimental groups.

The care and maintenance of animals were as per the approved guidelines of the Committee for Control and Supervision of Experiments on Animals. Requirements of environment and housing facilities of laboratory animal comply with national standard, P. R. China (GB 14925–2001). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC, China) and the experimental animal protocols were approved by the Institutional Animal Ethical Committee of Tianjin Institute of Pharmaceutical Research, Tianjin, China.

4.2. Chemicals and immunoglobins

Mouse IgG was purchased from Beijing Chengwen Immunochemistry Laboratory (Beijing China) and stored at 4 °C. Horseradish peroxidase con-

jugated goat anti-mouse immunoglobin (IgG-HRP) and 3,3,5,5-tetramethyl benzidine (TMB) were purchased from Invitrogen and stored at 4 °C. Normal saline was provided by Otsuka Pharmaceutical Co., Ltd. China (Tianjin China). Cyclophosphamide for Injection was provided by Jiangsu Hengrui Medicine Co. Ltd.(Jiangsu China).

4.3. Bacterial strain, plasmid, and cancer cell line

Escherichia coli BL21 (DE3) was purchased from Novagen and grown on Luria–Bertani (LB) medium. The expression vector pET-28a was from Novagen. Recombined plasmids pET-28a-SEC2 and pET-28a-SAM1 containing the full length of SEC2 cDNA and the full length of 2M-118 cDNA, respectively, were constructed in our lab (Xu et al. 2011). S180 sarcoma cells were gifted by the Cancer Drug R&D Center of Tianjin Institute of Pharmaceutical Sciences.

4.4. Vectors construction, protein expression and purification

Polymerase chain reactions (PCR) were performed using recombined plasmids pET-28a-SEC2 and pET-28a-2M-118 as templates by a sense primer (5'- TAC CAT GGA GAG TCA ACC AGA -3') and an antisense primer (5′- TCG CTC GAG TTA TCC ATT CTT TGT TG -3′). The PCR fragments were digested by *Nco* I and *Xho* I, and ligated into plasmid pET-28a digested by the same enzymes to construct the expression vectors for rSEC2 and 2M-118. Both rSEC2 and 2M-118 used in this study did not have His-tag purification label, which was different from our previous study (Xu et al. 2011). The constructed plasmids were then transformed into *E.coli* BL21 (DE3), and verified by DNA sequencing.

The transformed BL21 (DE3) was cultured in LB medium supplemented with 50 ìg/mL kanamycin at 37 °C. The protein expression was induced with 1.0 mM IPTG for 4 h at 30 °C. The cells were harvested and sonicated on ice, and the solution was clarified by centrifugation at 12,000 × g for 30 min. The supernatant was collected and loaded onto the SP Sepharose FF cation exchange column pre-equilibrated with 10 M phosphate buffered saline (PBS) with pH 5.6. The target protein was eluted by PBS with gradient from pH 5.6, 10 M to pH 7.0, 0.3 M. The elution was dialyzed in 5 M Tris-HCl with pH 8.6 and loaded onto the Q Sepharose FF anion exchange column pre-equilibrated with 5 M Tris-HCl with pH 8.6. The target protein was eluted by Tris-HCl with gradient from pH 8.6, 5 M to pH 7.0, 0.3 M. Then the purified protein was dialyzed in 0.15 M PBS with pH 7.4 containing 10% glycerin, and stored at −20 °C.

4.5. Murine spleen lymphocyte proliferation assay

The T lymphocyte proliferation assay was performed by an MTT method. A spleen from a 6- to 8-week-old female BALB/c mouse was isolated and mashed into splenocyte suspension in Hanks solution aseptically. The cell suspension was then added with 1 mL of erythrocyte lysis buffer and maintained for 1–2 min at room temperature to cleave red cells. 5 mL of PRMI-1640 supplemented with 10% fetal bovine serum was added to stop the lysis and the cell suspension was centrifuged at 1000 rpm for 10 min, and after removing the supernatant, the cells were suspended in RPMI-1640 supplemented with 10% fetal bovine serum and the cell density was adjusted to 1×10^7 cells/mL. 100 μL of 1×10^7 cells/mL splenocytes in RPMI 1640 medium containing 10% fetal bovine serum was seeded into the well of a 96-well microtiter plate. Serial 10-fold dilutions of wild type recombinant SEC2 (rSEC2) and 2M-118 were added to each well in triplicate, respectively, starting with 10000 ng/mL to 1.0 ng/mL. The RPMI 1640 medium served as the negative control. The splenocytes were incubated at 37 °C in 5% CO_2 for 48 h, which was followed by adding 20 μL of 5 mg/mL methyl thiazol tetrazolium (MTT) in PBS to each well, and then the plate was placed at 37 °C in 5% CO_2 for 4 h. The supernatant was removed after the plate was centrifuged at 500 × g for 10 min. The pellets were dissolved in 150 μL of dimethyl sulfoxide (DMSO), and optical densities (ODs) was measured on a microplate reader at 570 nm with 630 nm as a reference wavelength. The degree of proliferation is expressed as "proliferation index (PI)", which is the ratio of optical density value of the test group to that of the negative control.

4.6. Antitumor test in vivo

S180 cell suspension for inoculation was prepared from ascites of the cancer-bearing mice aseptically and diluted with RPMI-1640 containing 10% fetal bovine serum. 0.2 mL of the 1×10^7/mL S180 cell suspension was inoculated subcutaneously into the right axilla and the operation was finished within 60 min from withdrawing ascites from the cancer-bearing mice.

The BALB/c mice (female) were given 2M-118 intravenously at doses of 5, 10, 20 mg/kg at 24 h following tumor cell inoculation, respectively. Normal saline served as negative control and cyclophosphamide as positive control. The low and the medium doses were administered in three fractions, each of which was injected i.v. every two days, and the high dose was given for one time. All the mice were sacrificed on the 20th day after the first dosing, and the tumors were isolated and weighed. Tumor growth inhibition rate (TGI) was calculated by the formula TGI = (1-T/C) × 100%, where T was the average tumor weight of the mice in the test groups, and C was the average tumor weight of the mice in the normal saline group.

4.7. Immunogenicity test

The BALB/c mice were given intravenously 2M-118 at doses of 3 mg/kg and 10 mg/kg twice a week for four weeks, respectively. Normal saline was used as negative control. Serum samples were obtained from the mice (half male and half female) on days 14 and 28, and on the fifteenth day (recovery day 15) that followed the last administration, respectively. All the samples were kept at -20 °C until analyzed for antibody titer.

In antibody assay, the levels of anti-2M-118 were quantitatively measured by an indirect ELISA method. The wells of a 96-well microtiter plate were coated with 10 μg/mL 2M-118 in 100 μL of PBS, and 10 μg/mL mouse antibody IgG as the positive control. The plate was kept at 4 °C overnight. After antigen coating, the wells were blocked with 2% BSA at 37 °C for 2 h. The sera were serially diluted in PBS containing 0.2% Tween 20 and 100 μL of each dilution was added into triplicate wells. Then the plate was incubated at 37 °C for 1 h. After the wells were washed three times with washing solution, the bound antibody was determined by adding 100 μL of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG into each well. The plate was incubated at 37 °C for 1 h and washed with washing solution three times again. Each well was filled with 100 μL of TMB and left to react at 37 °C for 20–30 min. The reactions were stopped with 100 μL of 2 mol/L H_2SO_4, and OD values were measured at 450 nm. Antibody titer is defined as the maximum dilution ratio of a serum sample at which the OD value of the diluted sample is 2.1 times greater than that of the negative control. The level of anti-2M-118 was evaluated by the logarithm of antibody titer, and data were expressed as means ± standard deviations (lgT ± SD).

The pooled anti-sera from the mice that had received 3 mg/kg rSEC2 or 2M-118 twice a week for 28 days were used to evaluate the immunogenicity of 2M-118. In the wells of a 96-well microtiter plate, 0.9 μg/mL 2M-118 and rSEC2 in 20 μL normal saline were mixed with the 40 μL of pooled sera diluted 10-fold, respectively. The sera from the mice that had received normal saline were used as negative control. After a two-hour binding of the antibodies in the sera to 2M-118 at 37 °C, the wells were added with 100 μL of 1×10^7 cells/mL murine spleen cells. Afterwards, the plate was put at 37 °C in 5% CO_2 for 72 h. The proliferation of murine splenocytes was measured with an MTS method. Proliferation index (PI) represented the results of splenocyte proliferation, and data were shown as "the means of triplicate determinations ± standard deviations".

4.8. Statistical analysis

Kruskal-Wallis H and Mann–Whitney U nonparametric tests were applied to determine the significance of the differences in logarithmic titers of anti-2M-118 between the control and experimental groups. One-way ANOVA was used for comparison of the differences in antitumor effects of 2M-118, and Student test was used for statistical analysis of neutralizing activity of anti-sera against 2M-118 and rSEC2. P value of less than 0.05 was considered to be statistically significant.

Acknowledgments: The authors thank Professor Zhi Yao and Professor Huiqiang Li of Immunology Department, Tianjin Medical University for their great support to this study. We also give our great thanks to Dr Dongsheng Xiong of Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College and Prof Renjie Hu of Tianjin Institute of Pharmaceutical Sciences for their contributions to this work.

This work was supported by a grant from the National Science & Technology Major Specific Projects of China for "Significant Creation of New Drugs" (2009ZX09103–692).

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