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A Novel Simple Method to Purify Recombinant Soluble Human Complement Receptor Type 1 (sCR1) from CHO Cell Culture

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Abstract The human complement receptor type 1 (CR1, C3 b/C4b receptor) is a polymorphic membrane glycoprotein expressed on human erythrocytes, peripheral leukocytes, plasma and renal glomerular podocytes, which consists of transmembrane and cytoplasmic domains with 30 repeating homologous protein domains known as short consensus repeats (SCR). CR1 has been used as an inhibitor for inflammatory and immune system for the past several years. Recently, it is reported that CR1 was found to suppress the hyper-acute rejection in xeno-transplantation and can be used to cure autoimmune diseases. A soluble form of CR1, called sCR1, is a recombinant CR1 by cleaving the transmembrane domain at C-terminus and has been expressed in Chinese Hamster Ovary (CHO) cells. Several purification methods for sCR1 from CHO cells have been reported, but most of them require complicated steps at high cost. Moreover, such methods are mostly performed under the pH condition apt to denaturing sCR1 and causes sCR1 losing its activity. We here report a rapid and efficient method to purify sCR1 from CHO cell. The new method consists of a two-stage of cell culture by cultivating cells in serum medium followed by serum-free medium, and a two-stage of column purification by means of heparin and gel filtration column chromatography. By using this novel method, sCR1 can be purified in a simple and effective way with high yield and purity. Furthermore, the purified sCR1 was confirmed to retain its activity to suppress the complement activation *in vivo* and *ex vivo*.

Keywords: sCR1, two-stage cell culture method, two-stage protein purification method, C3b, factor I, necrosis

INTRODUCTION

The complement receptor type 1 (CR1) is a polymorphic membrane glycoprotein expressed on human erythrocytes, monocytes, macrophages, granulocytes, follicular dendritic cells, glomerular podocytes, and B- and T-lymphocytes. Since CR1 can bind complements C3b and C4b, it is regarded as a receptor of C3b/C4b. The main sequence of CR1 has been determined previously [1-3], which comprises a series of tandemly arranged 30 short consensus repeats (SCR), and each SCR contains 60 to 70 amino acids. In general, CR1 consists of four long homologous repeats (LHR) formed by seven SCR. The four LHRs are LHR-A, -B, -C and D. LHR-A contains a C4b-binding domain near the N-terminus, LHR-B and -C contain C3b-binding domains, and LHR-D has a cytoplasm tail near the C-terminus which was followed by two additional SCRs: a transmembrane domain of 25 amino acids, and a cytoplasmic domain of 43 amino acids with a carboxyl terminus.

Since CR1 can serve several regulatory functions such as co-factor activity with factor I, inactivation of bursting oxidization of neutrophil cells, hemolytic reaction with complement, and processing of C3 to C3a and C5a, it is widely used for clinic application. However, to extract CR1 from human cells requires a large quantity of erythrocytes and/or macrophages, it is impossible to obtain so many fresh human cells frequently. Weisman *et al.* [4] reported a construction of CR1 plasmid (pBSCR1 c/pTCSgpt) by cloning a soluble CR1 gene to plasmid pTCSgpt, in which a stop codon was inserted before the transmembrane and cytoplasmic domains. The plas-mid was then transfected to Chinese Hamster Ovary (CHO) cells and a 220 kDa of soluble CR1 (sCR1) could be expressed and secreted by CHO cells. The purified protein was confirmed to retain its well-known functions similar to those of cell-bound receptor CR1 *in vivo* [4]. Furthermore, its activity was also confirmed in the animal models with diseases, such as reversed passive arthus reaction [5], ischemic myocar-

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dium [4], reperfusion injury of ischemia [6], autoimmune disease and hyper-acute rejection for xeno-transplantation [7-10]. Therefore, sCR1 is an important regulatory protein to complement and can be administered to patients during xeno-transplantation to prevent acute immune rejection caused by the activation of complement.

Several methods to purify sCR1 from CHO cells have been reported [5,11-14], but difficulty still remains because of the complicated steps using multi-columns of chromatography at high cost and long operation duration. The most vital problem is the denaturation of sCR1 during the long term of multiple column operation which causes protein losing its activity. In order to solve these problems, we tried to develop a novel method by combining a high yield of cell culture method and a simple protein-purification method. By using this method, a high yield and high purity of sCR1 protein can be obtained rapidly. Moreover, sCR1 protein purified by this method is confirmed to retain its full structure and high activity both *in vitro* and *ex vivo*.

MATERIALS AND METHODS

Cells and Culture Medium

The high yield of cell culture method comprises a two-stage cell culture of CHO expressing sCR1. The first stage is a stage to grow cells in the medium with fetal bovine serum, and the second stage is a stage to produce sCR1 protein by cells in a serum-free medium. For the first stage of cell growth culture, cells were cultured in various medium such as α-MEM (Gibco BRL), F-12 (Sigma), DMEM/F-12 (Sigma), F-10 (Sigma) and RPMI 1640 (Sigma) supplemented with 10% FBS (Sigma), 500 nM methotrexate (MTX; Sigma), L-glutamine, penicillin (100 U/mL; GIBCO BRL), streptomycin (100 μg/mL; GIBCO BRL) and $NaHCO_3$ (2.2 g/L; Wako). Additionally, medium without deoxyribonucleoside and ribonucleoside were especially chosen for the cell culture of CHO. For the second stage of protein production, cells were cultured in various serum-free medium such as ASF104 (Ajinomoto; without albumin), DMEM/F-12 (1:1 and Sigma), α-MEM (Gibco BRL), F-12 (Sigma), α-MEM/ F -12 (1:1, Gibco BRL and Sigma) and MCDB302 (ICN), supplemented with the 500 nM methotrexate. All cell culture was performed in the incubator with 5% CO_2 at 37°C.

Two-stage Cell Culture Method

Cell line of CHO (CRL-10052) was purchased from ATCC (American Type Culture Collection) and was cultured under the conditions according to the ATCC's instructions. Cells were cultured in 150 mm dish (25 mL volume) until confluent, and then detached from dish with a solution of 0.25% trypsin-1 mM EDTA-4Na (Gibco BRL) in phosphate buffered saline (PBS; Nissui), and centrifuged at 800-1,000 ×*g* for 5 min. After that, cells were washed with PBS three times and then seeded on 150 mm dish at a concentration of 2.0×10^6 cells/mL in a fresh medium supplemented with 10% FBS. When cells reached confluent at a concentration of about 2.5×10^7 cells/mL, three washes with PBS were conducted followed by changing the medium with serum-free medium. After 36 h, the supernatant of cell culture was collected and fresh medium was added to the dish to cultivate cells for another 36 h. Supernatant was collected and pooled together with the first supernatant. Subsequent to centrifugation at 800-1,000 ×g for 5 min, the supernatant was 0.45 μm filter (Millipore). The quantity and quality of sCR1 in the filtrated sample were determined by subjecting the sample to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad) under reducing condition, subsequent Coomassie brilliant blue (CBB) staining [15-18].

Two-stage Purification Method

The method of two-stage purification comprises two phases of chromatography. The first phase is to bind sCR1 from filtrated sample by affinity column chromatography, and the second one is to separate sCR1 from the first phase by gel chromatography. For affinity column, heparin-Sepharose CL-6B (Pharmacia Biotech) swelled in distilled water was packed in a column (1.5 ϕcm ×15 cm). The column was then washed with distilled water and equilibrated with equilibrated buffer (20 mM Tris-Cl buffer (pH 7.4) containing 0.05% CHAPS (ICN)). Subsequently, 1 L of the filtrated sample was loaded to heparin column and the pass through fractions were collected. The column was washed and the bound protein was eluted with a linear gradient of 0.15-1 M NaCl. Finally, the column was washed three times with 0.1 M Tris-Cl (pH 8.5) and 0.1 M sodium acetate (pH 5.0) buffer containing 0.5 M NaCl, and re-equilibrated with equilibrated buffer. Each eluted fraction sample was subjected to SDS-PAGE and subsequent CBB staining.

The second phase of purification was carried out by means of HPLC gel filtration column chromatography. Samples eluted from heparin column were pooled together, and injected to HPLC gel filtration column chromatography (TSK gel G3000SWXL; TOSOH). Tris-Cl buffer (pH 7.4) containing 0.05% CHAPS was used as elution buffer. 1-2 mL eluted sample was recovered from each injection, and subjected to SDS-PAGE subsequent CBB staining. The recovered sample was further condensed to half volume by means of centrifuge filter device (Centri-plus; MW cut-off 100 kDa; Millipore) and dialyzed through molecular-porous membrane tube (MW cut-off 12-14000; Spectra/ Por exclusion) for further biochemical assay.

Western Blot Analysis

The purified sCR1 protein was subjected to four separative SDS-PAGE under non-reducing condition and then blotted to nitro-cellulose membrane (0.45 μm: Amer-

sham Pharmacia Biotech) at a constant current of 0.6 mA/cm^2 for 45 min using a wet blotter. Subsequently, each membrane was blocked with 5% skimmed milk (Snow Brand Milk Products) in PBS-T buffer (0.05% Tween 20 (Wako) in PBS). Four kinds of primary mouse anti-human CR1 monoclonal antibodies (E11; PHARMINGEN, To5; DAK, J3D3; BECKMAN COULTER, and KuN241; Neo Makers) were used. Each primary antibody was diluted to 500 fold and added to membrane at a concentration of 1 μg/mL in PBS-T buffer. After that, the four membranes were incubated for 2 h, followed by three washes in PBS-Tween solution. The membrane was then incubated for 1 h with 1,000-fold diluted horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Life Science) and washed with PBS-T buffer three times. The site of the antibody-antigen complex on the membrane was visualized by the reaction of ECL kit solution (Western blotting detection reagent; Amersham Life Science) [19,20].

Assay for the Co-factor Activity of sCR1 Reacting with Factor I

sCR1 has been known to have the co-factor activity reacting with factor I so as to degrade complement C3b to iC3b *in vivo*. Since C3b is unstable *in vitro*, we used C3ma, a methylamine treated C3, to replace C3b for the *in vitro* assay. The reaction was conducted by reacting C3ma (25 μg) with sCR1 (8 μg) as well as factor I (0.5 μg) in 100 μL distilled water at 37°C for 8 h. The reaction mixture was then subjected to SDS-PAGE subsequent CBB staining [21,22].

Assay for the Suppression Effect of sCR1 on the Necrosis of PA-6 Cells

In order to investigate the suppression effect of sCR1 on complement activation, PA-6 cell, a stroma cell derived from mouse skull bone marrow, kindly gifted from RIKEN, was used for this assay. PA-6 cells were seeded in 24 well plates and allowed to grow in the medium of α-MEM with 10% FBS for one day. After three washes with PBS, cells were added with 900 μL of serum-free medium (α- MEM) as well as 100 μL of PBS solution containing sCR1and normal human serum (NHS) at a ratio of 1:9, or 100 μL of PBS solution containing sCR1 and heat-inactivated NHS at a ratio of 1:9. After 1 h incubation at 37°C, the suppression effect on the necrosis of cells was observed via microscope (Nikon DP50-WMED) following the methods described previously [23-25].

RESULTS AND DISCUSSION

Two-stage Cell Culture Method

Several methods to purify sCR1 from CHO cells have been reported [5,11-14], but difficulty still remains due to the complicated steps, long operation duration and high cost of multi-columns of chromatography. Most of the CHO cells used to produce sCR1 have been cultured in medium with serum so as to grow cells as many as possible. However, serum itself contains various amino acids and proteins which require many complicated steps to be separated and removed from the product. Moreover, such steps usually cause the loss of product and reduce the yield of the desired protein. In order to solve these problems, we developed a two-stage cell culture method by dividing cell culture into two stages: cell growth stage and protein-producing stage. In the cell growth stage, serum was added to the medium, while no serum was added in the protein-producing stage, since the latter stage is conducted in serum-free medium, no many undesired proteins exists in the medium. It is easy to recover the desired sCR1 protein from the supernatant of such cell culture, and no further multiple steps are required to remove the undesired

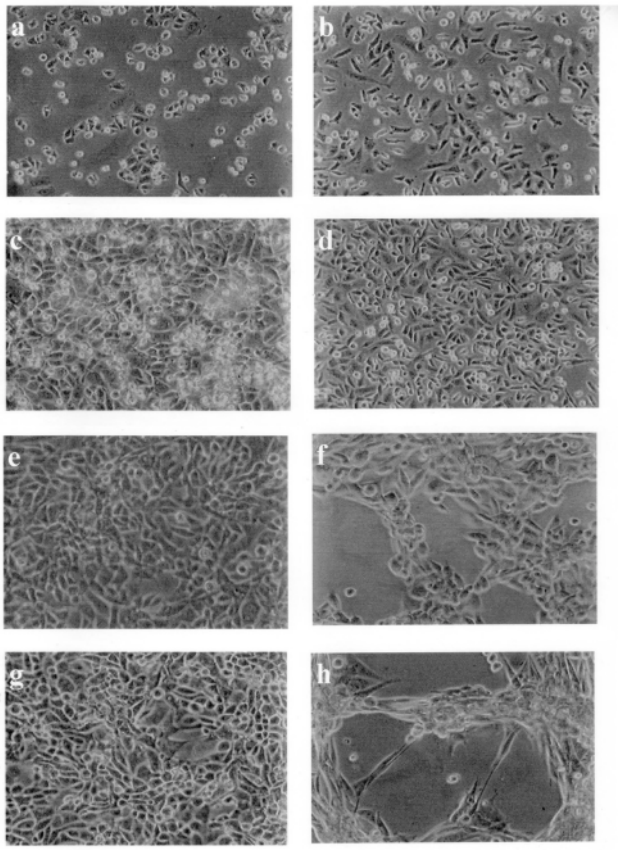

Fig. 1. Phase contrast micrograph of CHO cells in the two-stage of cell culture. For cell growth phase, cells were cultured in α-MEM medium with 10% FBS at (a) 0 hr and (c) 48 h, and in DMEM/ F-12 medium with 10% FBS at (b) 0 h and (d) 48 h. For protein-producing stage, cells shifted from α-MEM medium to ASF104 medium were cultured at (e) 24 h and (g) 72 h, and cells shifted from DMEM/F-12 medium to ASF104 medium at (f) 24 h and (h) 72 h. (magnification ×100). The seeding concentration of cell is 2.0×10^6 cells/mL, and the confluent cell concentration is 2.5×10^7 cells/mL.

impurities. Fig. 1 shows cell morphology in cell growth stage and protein-producing stage. In the cell growth stage, cells were observed growing well in both α-MEM and DMEM/ F-12 with 10% FBS after seeding on dish (Fig. 1(a), (b)) and after 48 h (Fig. 1(c), (d)). However, when cell culture was shifted to protein-producing stage with serum-free medium, only those pre-cultured in α-MEM reached confluent quickly (Fig. 1(e), (g)) while those in DMEM/F-12 medium changed cell morphology to elongate and could not reach confluent even after 72 h (Fig. 1(f), (h)). Moreover, cells pre-cultured in DMEM/F-12 medium were found detaching from dish in protein-producing stage (data not shown), which suggests that cell anchorage of CHO may play an important role for producing sCR1. The reason for this phenomenon is not clear yet but cell-cell interaction could be a main reason. As anchorage caused CHO cells staying stable with the neighbor cells, and therefore could enhance cell-cell interaction and resulted in a satisfactory protein production. Other medium such as RPMI1640 and F-10 were also investigated, but no medium could grow cells better than α-MEM after shifting to protein-producing stage. The rapid cell growth in α-MEM medium probably is due to its enhancement on cell anchorage. As a result, α-MEM with 10% FBS is considered the optimum cell growth medium for CHO cells.

Various serum-free medium for protein-producing stage such as α-MEM, α-MEM/ F-12, F-12, DMEM/F-12, MCD302 and ASF104 were also compared. Fig. 2 shows the SDS-PAGE analysis of samples collected from various media. It is remarkable that ASF104 produced high yield of sCR1 (40 μg/mL) without many impurities (Fig. 2(f)) as compared to other medium (Fig. 2(a)-(e)). ASF104 is reported to be suitable for culturing both suspending and adhesive cells such as hybridoma and hepatocytes [26,27]. Our results show that ASF104 is also a promising medium for CHO cells to produce sufficient sCR1. It is obvious that ASF104 can not only enhance cells anchorage but also prevent cells from producing unnecessary proteins except for those proteins useful to cell anchorage such as fibronectin, vitoronection and laminin. This fact is consistent with that reported previously [26]. Therefore, ASF104 is considered an optimum medium for sCR1 protein-producing stage.

In addition to the medium, other culture conditions such as culture duration were also investigated, and the result showed that 48 h is the optimum duration for cell growth stage and 36 h for protein producing stage.

Taken together, α-MEM with 10% FBS can grow CHO cells to confluent rapidly and the serum-free ASF104 can activate cells to secrete a high yield of sCR1 with less undesired proteins. Our method of cultivating cells in two stages not only overcomes the difficulties of previous methods such as long terms of cell culture and high cost of serum medium, but also reduces the impurities in cell culture significantly. By using this two-stage cell culture method, the further steps for protein purification can be simplified and high cost of multiple purification columns can also be saved.

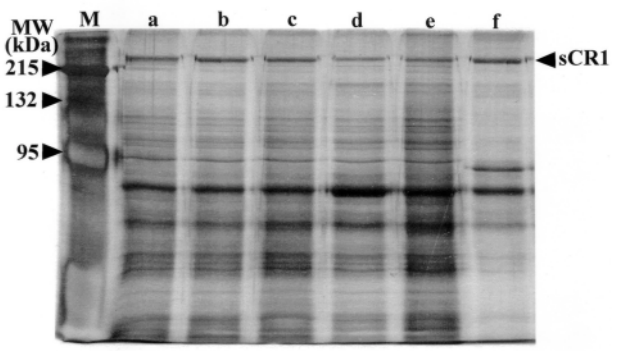

Fig. 2. Silver stained SDS-PAGE analysis of the supernatant of cell culture in (a) α-MEM, (b) α-MEM/F-12, (c) F-12, (d) DMEM/F-12, (e) MCDB302 and (f) ASF104. 20 μL sample each was loaded on gels under reducing condition. Lane M indicates molecular weight marker proteins. MW is the abbreviation of molecular weight, and the sCR1 concentration at lane f is 40 μg/mL.

Two-stage Purification Method

The method of two-stage purification comprises two phases of chromatography. The first phase is to bind sCR1 from filtrated sample by affinity column, and the second one is to separate sCR1 by gel chromatography. In the first phase, heparin column was used to remove the undesired impurities from the supernatant of cell culture. Heparin is a 3,000-10,000 Mr of glycosaminoglycan having a repeating structure of two glycosides consisting of uronic acid with either D-glucuronic acid or L-iduronic acid, and D-glycosamine. It has been reported that heparin has affinity with tyrosine kinase-like receptor such as heparin binding growth factor (HBGF), von Willebrand factor (vWF) [29] and many kinds of cytokine [30-33]. Moreover, it has also been found that heparin has specific affinity with CR1 [34-36]. In this study, heparin column was used to separate sCR1 from impurities by which sCR1 was bound and impurity protein was passed. Fig. 3 shows the SDS-PAGE analysis of each elution sample. Supernatant of cell culture was used as a control shown in lane a, and the sample passing through heparin column is shown in lane b. Lane c shows the heparin-bound protein eluted with 0.45 M NaCl. The results indicate that sCR1 (220 kDa) as well as the impurity protein (80 kDa) existed in the supernatant of cell culture (lane a), while no sCR1 can be detected in the pass through fraction (lane b). This fact suggests that heparin bound sCR1 effectively and completely. After eluting the bound protein with NaCl, a significant band of sCR1 (220 kDa) was detected (lane c) at a concentration of 400 μg/mL. It implies that heparin column not only bound sCR1 purification but also condensed sCR1 to a concentration 10 folds (400 μg/mL) higher than that obtained from cell supernatant (40 μg/mL) at lane f of Fig. 2. Therefore, heparin column is considered as a suitable column for the sCR1 purification. Various concentrations of NaCl

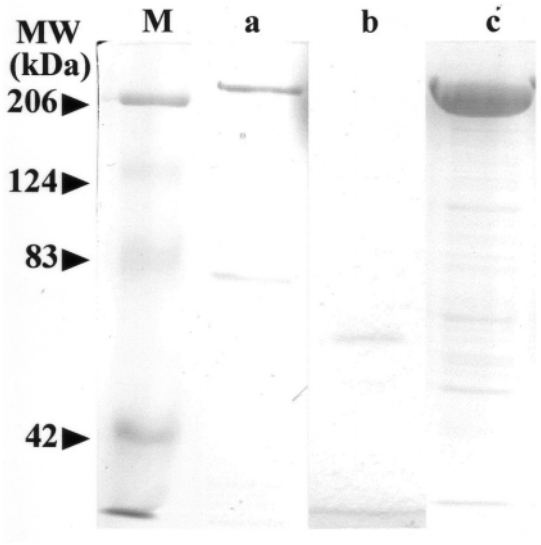

Fig. 3. CBB stained SDS-PAGE analysis of (a) supernatant of cell culture in ASF104, (b) sample passing through heparin column, and (c) sample eluted from heparin column with 0.45 M NaCl. 20 μL sample each was loaded on gel under reducing condition. M indicates molecular weight marker proteins. The sCR1 concentration at lane c is 400 μg/mL.

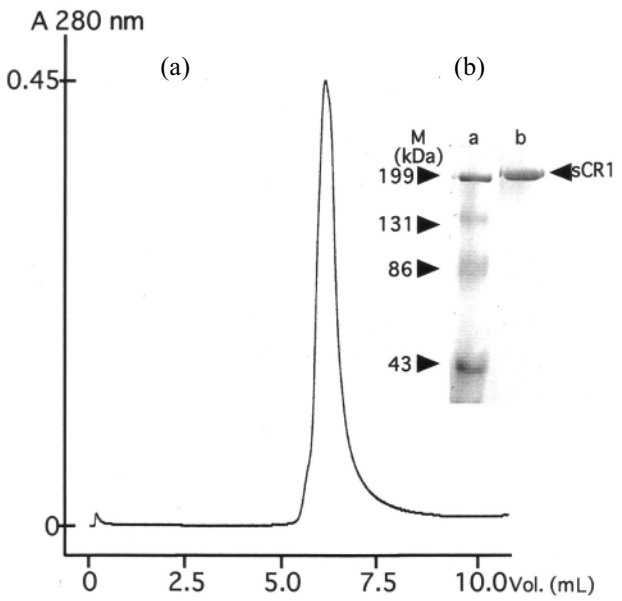

Fig. 4. (a) HPLC gel filtration chromatogram of sCR1 fraction in the second purification stage. One injection contains 500 μL sample. (b) CBB stained SDS-PAGE analysis of (a) eluted sample from gel filtration chromatography. 20 μL sample was loaded on gel under reducing condition. M indicates molecular weight maker proteins. The sCR1 concentration at lane a is 7.2 mg/mL.

for sCR1 elution have also been investigated, and the results showed that a concentration lower than 0.45 M could not elute sCR1 at all while the impurity protein of 80 kDa was increased (data not shown). As a result, heparin column has strong affinity with sCR1 and is able to separate sCR1 from impurity proteins and condense sCR1 effectively. Although some slight bands of impurities were found in lane c, it is worthy to notice that such bands might be formed by the high condensation efficiency of heparin column. In addition to sCR1, it has been described in the previous studies that heparin also has high affinity with proteins such as C4-binding protein [37-39], TSP (thrombospondin) [40,41], factor H [42], factor J [43], CR3 [44,45] and anaphylactic toxins [46]. It is likely that the slight bands in lane c is due to the high affinity of heparin which recognized and bound the above mentioned impurity proteins even in a very small amount. Under such conditions, further purification for sCR1 is necessary.

HPLC gel filtration column chromatography was adopted as the second column for further purification. Gel filtration can separate proteins by retarding small size protein and eluting large size protein quickly. Figure 4 shows the result of gel filtration chromatography by loading the sCR1 fraction collected from the first purification of heparin column. Only one significant peak was monitored after injection of sCR1 fraction (Fig. 4(a)), this result implies that all the impurities were removed and sCR1 was purified completely. SDS-PAGE analysis indicated only one band close to 220 kDa, suggesting that 100% purity of sCR1 is the final product (Fig. 4(b)). Therefore, high purity of sCR1 can be obtained by using this two-stage purification method.

As compared with the previous methods, our method provides a simple way to purify sCR1 with only two column chromatography. Instead, the previous methods require more purification steps such as repeating precipitation with ammonium sulfate and antibody affinity column [11-14]. Moreover, our method only takes several hour to complete protein purification whereas the previous methods require long term for purification which usually causes protein denaturation due to the fragile structure. One-step method using cation exchange column chromatography has been developed previously [5], but it requires long term of dialysis for buffer exchange before loading the sample to the column. Such a step not only takes a great deal of time but also requires much labor-consuming. On the other hand, our method suggests a strategy to shift cell culture from serum medium to serum-free medium, which provides an easy and high efficient way to purify the desired protein. In fact, our method succeeded in recovering 100% purity of sCR1 in a yield up to 7.2 mg/mL whereas the previous methods recovered lower purity of sCR1 (< 90%) in a yield as low as 100 μg/mL. Our method combining two-stage cell culture and two-stage protein purification can facilitate and economize the sCR1 production and purification remarkably

Western Blot Analysis

In order to confirm no denature or degradation occurred

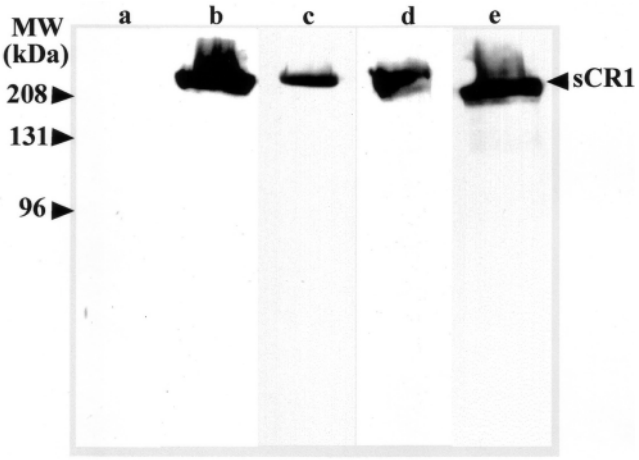

Fig. 5. Western blot analysis of the purified sCR1 protein (a) without primary antibody, and with primary monoclonal anti-sCR1 antibodies of (b) E11, (c) KuN241, (d) J3D3, and (e) To5. 20 μL sample each was subjected to SDS-PAGE under non-reducing conditions and visualized by ECL reagent. The primary antibodies were diluted to 500 fold and second antibody to 1000 fold in PBS containing 5% skimmed milk.

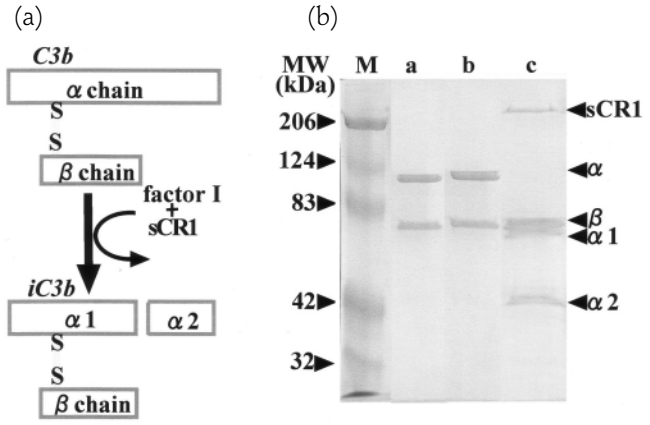

Fig. 6. (a) Pathway of C3ma (C3b) degradation in the presence of factor I and sCR1. (b) CBB stained SDS-PAGE analysis of the sCR1 co-factor activity. iC3b is formed by the activation of sCR1 and factor I. Reaction was conducted in (a) C3ma only, (b) C3ma and factor I, and (c) C3ma, factor I and sCR1 under reducing condition. M indicates molecular weight marker proteins.

during protein purification, the final product of sCR1 was subjected to western blotting under non-reducing condition with four kinds of antibodies E11, KuN241, J3D3 and To5. Each antibody binds the different domains of sCR1. For example, E11 binds LHR C and D, KuN241 binds LHR A and B, J3D3 and To5 bind LHR A, B and C [19,20]. The result in Fig. 5 indicating the purified sCR1 was bound by all kinds of antibodies suggests that our method can retain sCR1 in its full protein structure.

Assay for the Co-factor Activity of sCR1 Reacting with Factor I

CR1 has been known to have the co-factor activity reacting with factor I so as to degrade complement C3b to iC3b *in vivo* or *ex vivo* [21,22]. C3b contains two chains: α-chain (120 kDa) and β-chain (75 kDa), and iC3b is formed when the α-chain is degraded into two fragments: α1 fragment (73 kDa) and α2 fragment (43 kDa) in the presence of CR1 and factor I (Fig. 6A). Since C3b is very unstable *in vitro,* a similar complement C3ma, a methylamine treated C3, was used to replace C3b for the assay of the CR1 activity *in vitro*. Fig. 6(b) shows SDS-PAGE analysis result of the reaction mixture of C3ma and factor I, and the reaction mixture of C3ma, factor I and sCR1. C3ma is used as a control (lane a), two bands of α-chain and β-chain were detected. Similarly, lane b shows the same bands as those in lane a although factor I was added to C3ma. Such result implies that factor I is insufficient to degrade C3ma (C3b). After the addition of CR1 to the reaction mixture of C3ma and factor I, the α-chain of C3ma was found degraded into two fragments α1 and α2 (73 kDa and 43 kDa) while the β-chain (75 kDa) of C3ma remained as it was (lane c). Since excess sCR1 was added to reaction mixture, 220 kDa band of unreacted sCR1 was detected whereas factor I could not be detected due to its small amount. These results indicated that sCR1 protein purified by our method could retain its activity *in vitro* completely.

Assay of the Suppression Effect of sCR1 on the Necrosis of PA-6 Cells

In order to investigate the suppression effect of sCR1 on complement activation, PA-6 cell, a stroma cell derived from mouse skull bone marrow, was used for this assay. Since human serum comprises complements 1-9, which trigger the activation of complement pathways and attack target cells of xenograft. The complement pathway ends with the necrosis of target cells due to the attack by C5b-9 membrane attack complex (MAC) [23,24]. In order to determine whether the purified protein sCR1 retains its activity to bind complement C3b and/or C4b and suppresses the necrosis of cells by blocking complement pathway, sCR1 was added to a mixture of PA-6 cells and normal human serum (NHS). The results are shown in Fig. 7. Cells cultured in medium was used as a control (Fig. 7(a)), and cells cultured with inactivated NHS shows similar result as the control that all cells grew well, and maintained their normal morphology (Fig. 7(b)). This fact implies that the inactivated complements in the serum could not trigger any complement pathway. However, cells cultured with NHS revealing remarkable necrosis (Fig. 7(c)) implies that NHS triggered the activation of complement pathway and MAC attacked the cell membrane and caused cell death. On the other hand, when cells were cultured in the presence of NHS as well as the purified sCR1, cells showed normal morphology without any

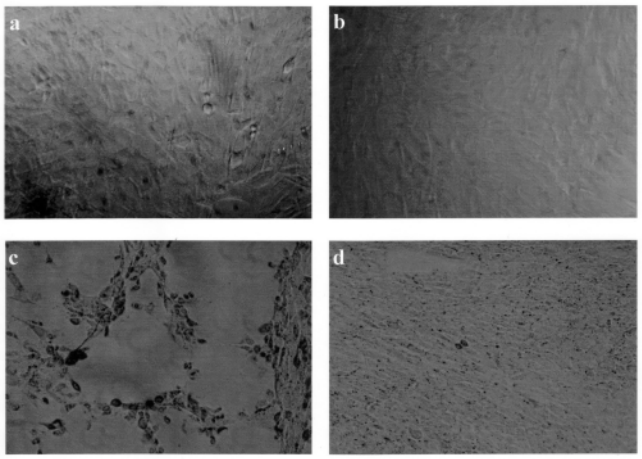

Fig. 7. Suppression effect of cell necrosis by sCR1 on PA-6 cells. Phase contrast micrograph of PA-6 cells in (a) α-MEM media (control), (b) in the presence of heat inactivated NHS, (c) in the presence of NHS, and (d) in the presence of NHS and purified sCR1 (magnification ×40).

necrosis although most of cells turn slim in their shape (Fig. 7(d)). These results proved that the purified sCR1 has an activity to bind C3b and C4b in NHS and plays its role to block the activation of MAC.

In conclusion, we have succeeded in developing a novel method to purify sCR1 protein from CHO cell culture in a high yield and high purity. Moreover, the purified protein can retain its full structure and activity *in vitro* and *ex vivo*. Taken together, our new method combining two-stages of cell culture and two-stages of protein purification provides significant advantage to produce and purify sCR1 in a simple and efficient way, such method can be applied to mass production in a large scale by means of bio-reactor in the future.

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