

The Soluble Form of the Cellular Prion Protein Enhances Phagocytic Activity and Cytokine Production by Human Monocytes Via Activation of ERK and NF- κ B

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The PrP^C is expressed in many types of immune cells including monocytes and macrophages, however, its function in immune regulation remains to be elucidated. In the present study, we examined a role for PrP^C in regulation of monocyte function. Specifically, the effect of a soluble form of PrP^C was studied in human monocytes. A recombinant fusion protein of soluble human PrP^C fused with the Fc portion of human IgG1 (designated as soluble PrP^C-Fc) bound to the cell surface of monocytes, induced differentiation to macrophage-like cells, and enhanced adherence and phagocytic activity. In addition, soluble PrP^C-Fc stimulated monocytes to produce pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6. Both ERK and NF- κ B signaling pathways were activated in soluble PrP^C-treated monocytes, and inhibitors of either pathway abrogated monocyte adherence and cytokine production. Taken together, we conclude that soluble PrP^C-Fc enhanced adherence, phagocytosis, and cytokine production of monocytes via activation of the ERK and NF- κ B signaling pathways.

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INTRODUCTION

The cellular prion protein (PrP^C) is a 32 kDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein found in specif-

ic structures of the cell membrane called lipid rafts (1). The N-terminal half of PrP^C is largely unstructured and is composed of a signal peptide and five octapeptide-repeat regions known to be binding sites for Cu²⁺ (2). The C-terminal half of PrP^C is folded into three alpha helices and two short beta-strands containing two oligosaccharide chains and a GPI- anchor at the end (3,4). Conformational conversion of PrP^C to a misfolded protein termed scrapie prion protein (PrP^{Sc}) is a major pathogenesis of transmissible spongiform encephalopathies (TSE), or the prion diseases, in animals and humans (5,6).

PrP^C is highly expressed in the central nervous system (CNS) and this is the major site of prion pathology (7). PrP^C is also expressed in many types of immune cells including lymphocytes, natural killer (NK) cells, monocytes, dendritic cells (DCs), macrophages, and follicular dendritic cells (8-12). In addition, PrP^C is known to be proteolytically shed by metalloproteinase (13,14), and a substantial amount of soluble PrP^C is found in the culture medium of splenocytes and in human serum (15). However, the function of membrane-bound PrP^C or soluble PrP^C in regulation of immune cells remains to be elucidated.

Physiological roles for PrP^C in monocytes and macrophages have been reported in several studies (16,17). Originally, PrP^C

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Abbreviations: PrP^C, cellular prion protein; PrP^{Sc}, scrapie prion protein; IKK, I κ B kinase; SEAP, secreted embryonic alkaline protease; HEK, human embryonic kidney; HEK 293E, HEK293 cells stably expressing the Epstein-Bar virus nuclear antigen 1; mRFP, monomeric red fluorescence protein; Syk, spleen tyrosine kinase; Pyk2, protein tyrosine kinase 2

was proposed to be a negative regulator of phagocytosis as macrophages from PrP^C-deficient mice showed greater phagocytic activity than macrophages from wild-type mice (18); however, recent studies with another line of PrP^C-deficient mice demonstrated that PrP^C enhanced phagocytic activity of macrophages (19,20). In addition, pseudopodium extension arrest and cell detachment were observed in macrophages from PrP^C-deficient mice (19). The effects of soluble PrP^C protein has also been studied (21). In this regard, it was found that soluble PrP^C protein activated several signaling proteins in mouse monocyte/macrophage cells, including ERK-1/2 and Akt kinase which are known to regulate phagocytosis, migration, and cytokine production by monocytes (21).

In the present study, we further investigated a role for PrP^C in regulation of monocyte function. In particular, the effect of a soluble form of PrP^C on monocytes was studied. To this end, we prepared a recombinant form of soluble human PrP^C as a fusion protein with the Fc portion of human IgG1 (designated as soluble PrP^C-Fc). We demonstrated that soluble PrP^C-Fc induced differentiation of monocytes to macrophage-like cells, and enhanced adherence and phagocytic activity of monocytes. In addition, soluble PrP^C-Fc stimulated monocytes to produce pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6. We also demonstrated that soluble PrP^C-Fc exerted its effects on monocytes via activation of the ERK and NF- κ B signaling pathways.

MATERIALS AND METHODS

Reagents

LPS was purchased from Invivogen (San Diego, CA). PMA was purchased from Sigma-Aldrich (St. Louis, MO). PD98059, SN50, and SB203580 were purchased from Calbiochem-Behring (La Jolla, CA). FITC-conjugated Abs against CD1a and CD11b, and control IgG were purchased from BD Biosciences (San Jose, CA). Abs against ERK-1/2, IKK, I κ B, and the phosphorylated form of each protein were purchased from Cell Signaling Technology (Beverly, MA).

Cells and cell culture

Human monocytic leukemia THP-1 cells were obtained from American Type Culture Collection (Manassas, VA). Human primary monocytes were obtained from PBMCs of normal donors. Primary monocytes were isolated from PBMCs with CD14 magnetic beads (Miltenyi Biotec, Auburn, CA). The purity of the isolated monocytes was verified with anti-CD14-

FITC Ab (BD Biosciences) staining and flow cytometric analysis. The purity of isolated human monocytes was typically 90 ~ 94%. THP-1 BlueTM cells, which are THP-1 cells with an NF- κ B-inducible secreted embryonic alkaline phosphatase (SEAP) reporter, were purchased from Invivogen. Cell culture was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM), HEPES (10 mM), and sodium pyruvate (1.0 mM).

Production of recombinant soluble human PrP^C-Fc

A recombinant form of soluble human PrP^C protein consists of amino acids 21~229 of human PrP^C fused with the Fc portion of human IgG1 at the C-terminus. Soluble PrP^C-Fc was expressed in HEK 293E cells and purified on a protein A-Sepharose column (Amersham Biosciences, Sunnyvale, CA) according to the manufacturer's instructions. Human IgG1 Fc without the PrP^C protein was also expressed and purified using the same methods, and was used as a negative control. The purified recombinant protein was dialyzed with PBS, analyzed by SDS-PAGE, and then analyzed with the Limulus Amebocyte Lysate Test Kit (Cape Cod, East Falmouth, MA) to determine the endotoxin level. The endotoxin level in the recombinant protein preparations was less than 10 EU/ml.

Soluble PrP^C binding assays to monocytes

Human primary monocytes were fixed with 4% paraformaldehyde for 10 min and blocked with 5% normal goat serum for 30 min. They were then incubated with control Fc or with soluble PrP^C-Fc at various concentrations, followed by labeling with FITC-conjugated anti-human IgG. Flow cytometry was performed using LSRII (BD Biosciences), and the data were analyzed using FlowJo software (TreeStar, San Carlos, CA).

Cell adherence assay and phagocytosis assay

Cell adhesion assay was performed as described previously (22). Briefly, ten thousand THP-1 cells were plated in each well of a 96-well culture plate and treated with soluble PrP^C-Fc for 30 min. The plate was then washed three times with PBS, and microscopic photographs were taken. The cells were fixed in methanol, stained with a 10% (v/v) solution of Giemsa (Sigma-Aldrich), and the number of adherent cells was evaluated over 10 fields. For each well, the ratio between the number of cells in 10 fields after plating (N_T) and the number of adherent cells in 10 fields after culturing and washing (N_A) was defined as the percent adherence ($N_A/N_T \times$

100). To measure phagocytosis, primary monocytes were differentiated for 48 h in the presence of soluble PrP^C-Fc. Then, cells were incubated for 6 h with *Escherichia coli* that expressed monomeric red fluorescent protein (mRFP). The cells were washed with cell culture medium without FBS, and the fluorescence intensity was assessed by flow cytometry.

Quantification of cytokine production

Primary monocytes were incubated with soluble PrP^C-Fc for 36 h. The culture supernatants were collected, and the concentrations of TNF- α , IL-1 β , and IL-6 were determined using the Quantikine Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. For inhibition of specific signaling pathways, PD98059 (20 μ M), SN50 (10 μ M), or SB203580 (10 μ M) were added to monocyte cultures 1 h before soluble PrP^C-Fc treatment.

Western blot

To analyze levels of ERK-1/2, IKK, I κ B, and the phosphorylated form of each protein, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM 4-nitrophenyl phosphate, 10 μ g/ml of leupeptin, 10 μ g/ml of pepstatin A, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluo-

ride). Cell lysates were centrifuged at 15,000 g for 5 min at 4°C. The supernatant was mixed with SDS sample buffer, boiled for 5 min, and then separated by 12% SDS-PAGE. The protein was transferred to nylon membranes by electrophoretic transfer. The membrane was blocked in 5% skim milk, rinsed, and incubated with a specific Ab in PBST overnight at 4°C. The membrane was rinsed four times in PBST, and incubated with 0.1 μ g/ml peroxidase-labeled secondary Ab for 1 h. After rinsing three times in PBST, specific bands were visualized by enhanced chemiluminescence.

SEAP reporter assay

THP-1 BlueTM cells contain a reporter plasmid that expresses a secreted embryonic alkaline phosphatase (SEAP) under the control of NF- κ B and AP-1 transcription factors. THP-1 BlueTM cells were incubated with soluble PrP^C-Fc for 48 h. To quantify secreted SEAP, the culture supernatant was incubated with QUANTI-BlueTM colorimetric assay reagent (Invivogen) for 24 h at 37°C. The OD at 655 nm was measured with a VERSAmix Tunable microplate reader (Molecular Devices, Toronto, Ontario, Canada). All assays were run in triplicate.

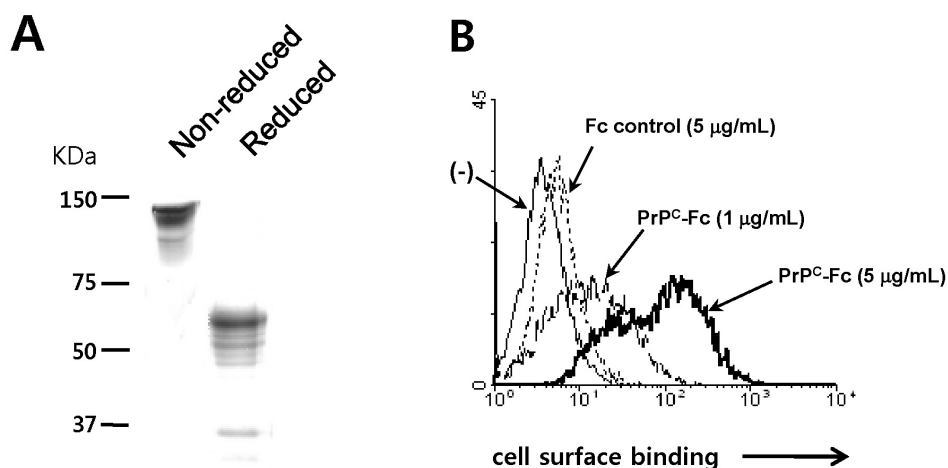


Figure 1. Preparation of soluble recombinant human PrP^C-Fc and its binding to monocytes. (A) SDS-PAGE analysis of purified soluble recombinant PrP^C-Fc protein. Soluble PrP^C-Fc consists of amino acids 21-229 of human PrP^C fused with the Fc portion of human IgG1. Protein samples were separated on a 4~20% gradient SDS-PAGE gel with or without reducing condition. The molecular weight of soluble PrP^C-Fc is 55~65 kDa in reducing condition and 120~140 kDa in non-reducing condition. (B) Human primary monocytes were fixed with 4% paraformaldehyde for 10 min and blocked with 5% normal goat serum for 30 min. They were then incubated with control Fc or with soluble PrP^C-Fc at the indicated concentrations, followed by labeling with FITC-conjugated anti-human IgG. Flow cytometric analysis shows the specific binding of soluble PrP^C-Fc to monocytes in a dose-dependent manner.

RESULTS

Soluble PrP^C-Fc binds to the cell surface of human monocytes

To study the function of soluble PrP^C on monocytes, we prepared a recombinant form of soluble human PrP^C (PrP^C-Fc). PrP^C-Fc consists of amino acids 21-229 of human PrP^C fused to the Fc portion of human IgG1 at the C-terminus. Soluble PrP^C-Fc was expressed in HEK293E cells, purified from culture supernatant, and analyzed by SDS-PAGE (Fig. 1A). First, we evaluated whether soluble PrP^C-Fc bound to the cell surface of human monocytes. Flow cytometric analysis showed

dose-dependent binding of soluble PrP^C-Fc to human monocytes (Fig. 1B). This result indicates that human monocytes express a putative binding partner of soluble PrP^C-Fc on their cell surface.

Soluble PrP^C-Fc induces differentiation of monocytes into macrophage-like cells

We next studied the effects of soluble PrP^C-Fc on monocytic cell function. We found that soluble PrP^C-Fc induced adherence of THP-1 monocytic cells within 30 minutes in a dose-dependent manner that was comparable to adherence induced by treatment with PMA (Fig. 2A). Notably, THP-1 cell

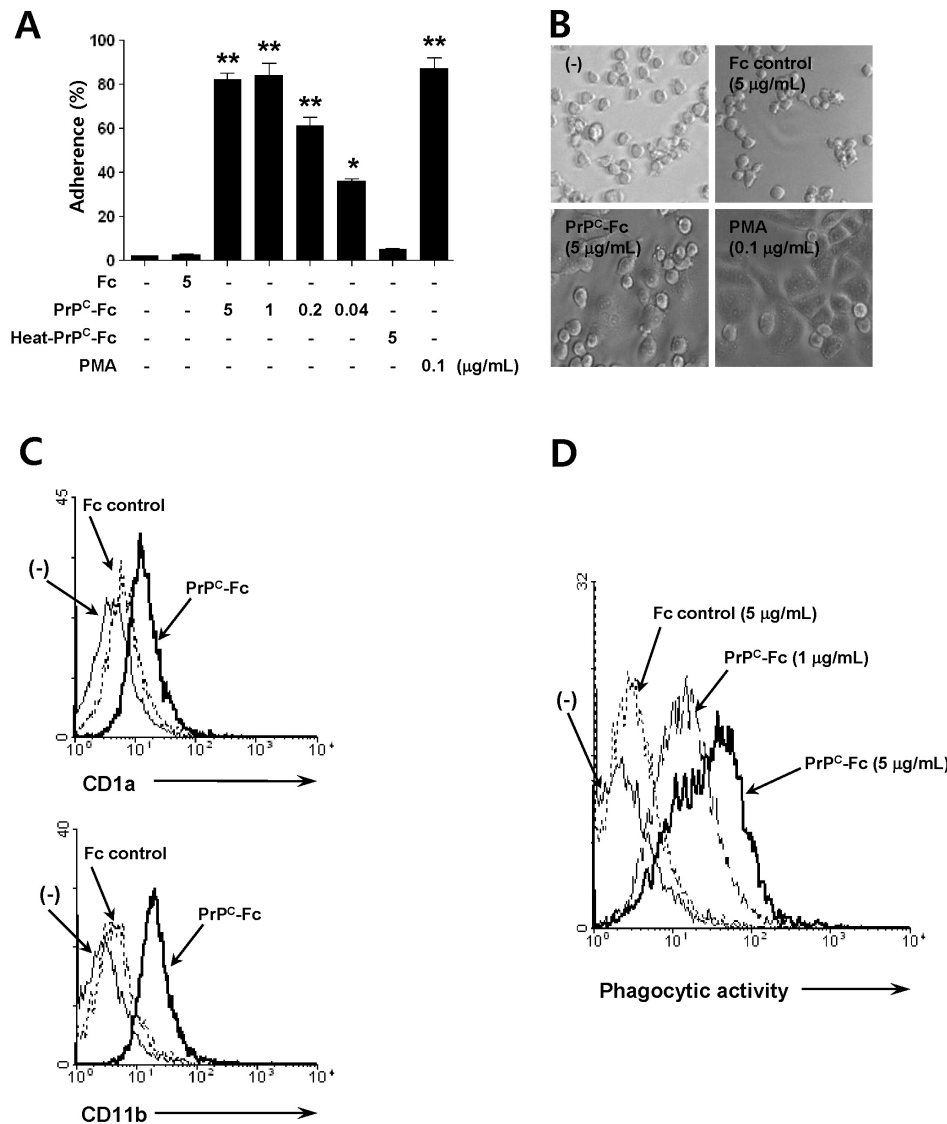


Figure 2. Differentiation of monocytes into macrophage-like cells following soluble PrP^C-Fc treatment. (A) THP-1 cells were treated with soluble PrP^C-Fc, heat-denatured (at 95°C for 30 min) PrP^C-Fc, or control Fc at the indicated concentrations for 30 min, and adherent cells were counted. Cell adherence was expressed as a percentage of the total number of cultured cells. The assay was performed in triplicate. Bar graphs represent the mean ± SEM. Statistical analysis was performed in comparison with untreated control. *p < 0.05; **p < 0.01. (B) Phase-contrast images of THP-1 cells treated with soluble PrP^C-Fc, control Fc, or PMA for 48 h (200× magnification). (C) Primary monocytes were cultured with soluble PrP^C-Fc or control Fc for 24 h followed by labeling with anti-CD11a-FITC or anti-CD11b-FITC and flow cytometric analysis. Similar results were obtained from three independent experiments. (D) Primary monocytes were cultured for 48 h in the presence of soluble PrP^C-Fc or control Fc and then incubated for 6 h with *E. coli* labeled with RFP. Flow cytometric data indicate the amount of phagocytosis of *E. coli* by monocytes. The data are representative of two independent experiments.

adherence was not induced by heat-denatured PrP^C-Fc (Fig. 2A). Moreover, after two days of culture with soluble PrP^C-Fc, THP-1 cells became flattened and exhibited a macrophage-like morphology, similar to that seen with PMA treatment (Fig. 2B). The expression of CD1a and CD11b on the cell surface of human monocytes was also significantly increased in response to soluble PrP^C-Fc (Fig. 2C). Next, we studied phagocytic activity by co-culturing human monocytes with mRFP-expressing *E. coli*. As measured by flow cytometry, we found that after 48 hours of culture with soluble PrP^C-Fc, phagocytic activity of human monocytes was significantly enhanced in a dose-dependent manner (Fig. 2D). Taken together, soluble PrP^C-Fc induces monocytes to acquire a macrophage-like phenotype including increased cell adherence, macrophage-like morphology, expression of cell surface markers such as CD1a and CD11b, and enhanced phagocytic activity.

Soluble PrP^C-Fc stimulates monocytes to produce pro-inflammatory cytokines

We investigated whether soluble PrP^C-Fc stimulated monocytes to produce pro-inflammatory cytokines. Human monocytes were cultured in the presence or absence of soluble PrP^C-Fc for 36 hours, and the concentrations of TNF- α , IL-1 β , and IL-6 were measured in culture supernatants. The production of TNF- α , IL-1 β , and IL-6 was stimulated by soluble PrP^C-Fc in a dose-dependent manner, but not by heat-denatured PrP^C-Fc (Fig. 3).

Soluble PrP^C-Fc activates signaling pathways for ERK and NF- κ B

Next, we studied whether soluble PrP^C-Fc activates intracellular signaling pathways in THP-1 cells. In particular, we investigated activation of ERK and NF- κ B, which are known to regulate differentiation of monocytes and production of pro-inflammatory cytokines (23). Following soluble PrP^C-Fc treatment, phosphorylation of ERK-1/2 was increased while the total amount of ERK-1/2 remained unchanged (Fig. 4A). A MEK-specific inhibitor, PD98059, abrogated ERK-1/2 phosphorylation induced by soluble PrP^C-Fc (Fig. 4A). In addition, soluble PrP^C-Fc increased phosphorylation of IKK and I κ B α in monocytes (Fig. 4B), suggesting that the NF- κ B pathway is also activated in response to PrP^C-Fc treatment. We further evaluated NF- κ B activity using a SEAP reporter assay in which THP-1 BlueTM cells secrete SEAP into the culture supernatant upon NF- κ B activation. We found that NF- κ B activity was in-

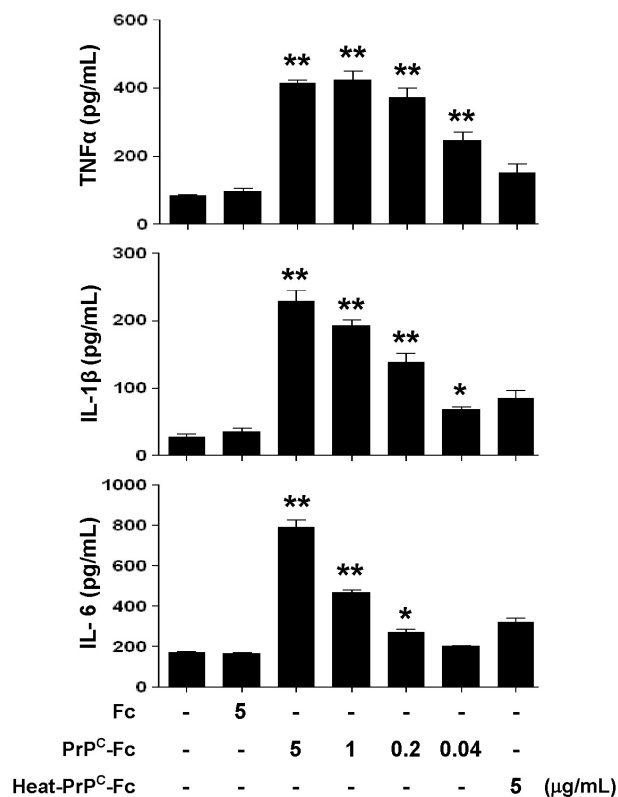


Figure 3. Cytokine production from monocytes stimulated by soluble PrP^C-Fc treatment. Primary monocytes were cultured with soluble PrP^C-Fc, heat-denatured PrP^C-Fc, or control Fc at the indicated concentrations for 48 h. The culture supernatant was collected, and the concentrations of TNF- α , IL-1 β , and IL-6 were determined with the Quantikine Assay Kit. The assay was performed in triplicate. Bar graphs represent the mean \pm SEM. Statistical analysis was performed in comparison with untreated control. * $p < 0.05$; ** $p < 0.01$. The data are representative of four independent experiments.

creased in response to soluble PrP^C-Fc in a dose-dependent manner, but not by heat-denatured PrP^C-Fc (Fig. 4C).

Monocyte adherence and cytokine production by soluble PrP^C-Fc depend on ERK and NF- κ B signaling

As soluble PrP^C-Fc activates ERK and NF- κ B in monocytes, we studied whether ERK or NF- κ B signaling is responsible for the downstream effects stimulated by this protein. To elucidate the role of each signaling pathway, we treated cells with specific signaling inhibitors including PD98059 to inhibit the ERK pathway and SN50 to inhibit the NF- κ B pathway, one hour before soluble PrP^C-Fc treatment. SB203580 was also used to inhibit the p38 signaling pathway. First, we found that THP-1 cell adherence induced by soluble PrP^C-Fc was

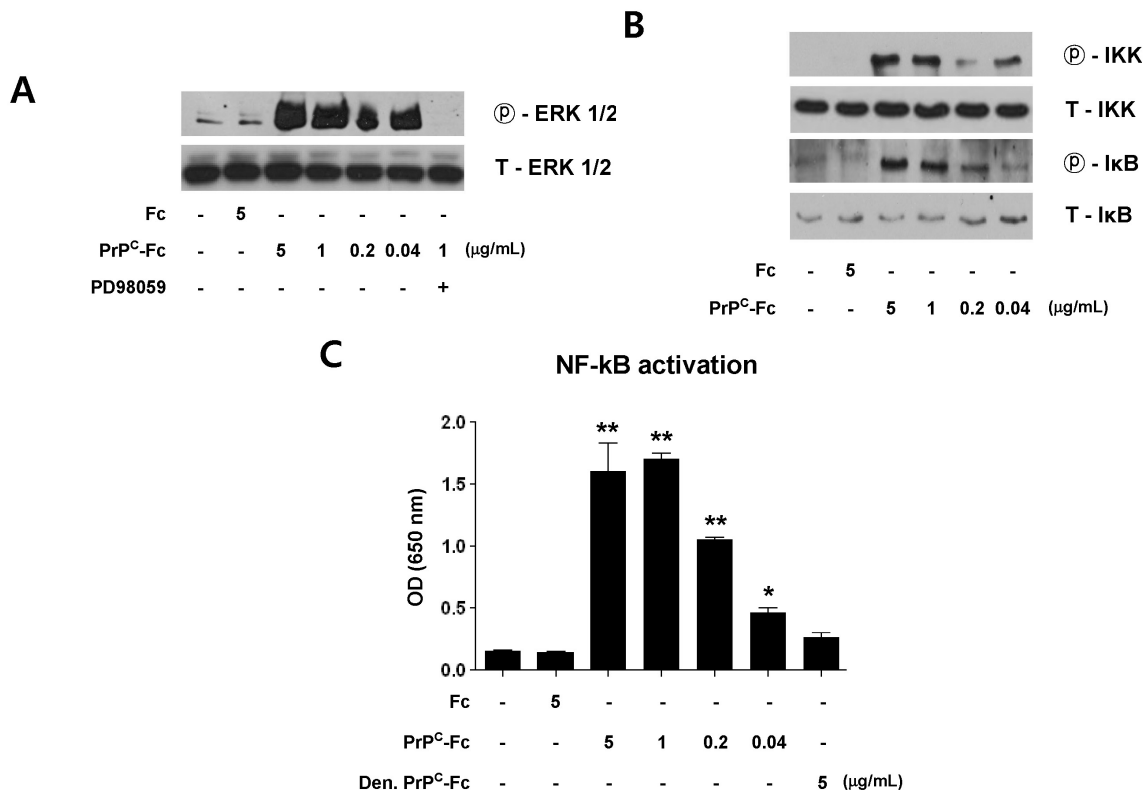


Figure 4. Activation of the ERK and NF- κ B signaling pathways in THP-1 cells stimulated by soluble PrP^C-Fc treatment. (A) THP-1 cells were treated with soluble PrP^C-Fc or control Fc at the indicated concentrations for 15 min. For inhibition of ERK signaling, PD98059 (20 μ M) was added to the THP-1 culture 1 h before soluble PrP^C-Fc treatment. Western blotting was performed with cell lysates to detect phosphorylated ERK-1/2 and total ERK-1/2. (B) THP-1 cells were treated with soluble PrP^C-Fc or control Fc at the indicated concentrations for 15 min. Western blotting was performed with cell lysates to detect phosphorylated IKK, total IKK, phosphorylated I κ B, and total I κ B. (C) THP-1 BlueTM cells, which secrete SEAP upon NF- κ B activation, were treated with soluble PrP^C-Fc, heat-denatured PrP^C-Fc, or control Fc at the indicated concentrations for 48 h. Secreted SEAP in the culture supernatant was quantified by a colorimetric assay as described in Materials and Methods, and the OD at 655 nm was measured with a microplate reader. The assay was performed in triplicate. Bar graphs represent the mean \pm SEM. Statistical analysis was performed in comparison with untreated control. * $p < 0.05$; ** $p < 0.01$.

significantly inhibited by PD98059 and by SN50, but not by SB203580 (Fig. 5A). Pro-inflammatory cytokine production by monocytes was also studied. Similar to cell adherence, soluble PrP^C-Fc-induced production of TNF- α and IL-1 β was significantly inhibited by PD98059 and by SN50, but not by SB203580 treatment (Fig. 5B). Taken together, soluble PrP^C-Fc induces adherence and cytokine production of monocytes via activation of ERK and NF- κ B.

DISCUSSION

PrP^C is a GPI-anchored protein found in lipid rafts of the cell membrane and expressed in immune cells as well as cells of the central nervous system. While PrP^C is known to be

proteolytically shed by metalloproteinase (13,14), and a substantial amount of soluble PrP^C is found in the culture medium of splenocytes and in human serum (15), the precise function of soluble PrP^C in immune regulation is unknown. In the present study, we investigated the roles for the soluble form of PrP^C in the regulation of monocytes and found that this molecule enhanced the adherence and phagocytic activity of these cells. Furthermore, soluble PrP^C stimulated monocytes to produce pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6.

The effect of soluble murine PrP^C protein on monocytes was previously examined. In this study, a recombinant murine PrP^C-Fc fusion protein was generated in a manner similar to the one we used to generate recombinant human PrP^C-Fc

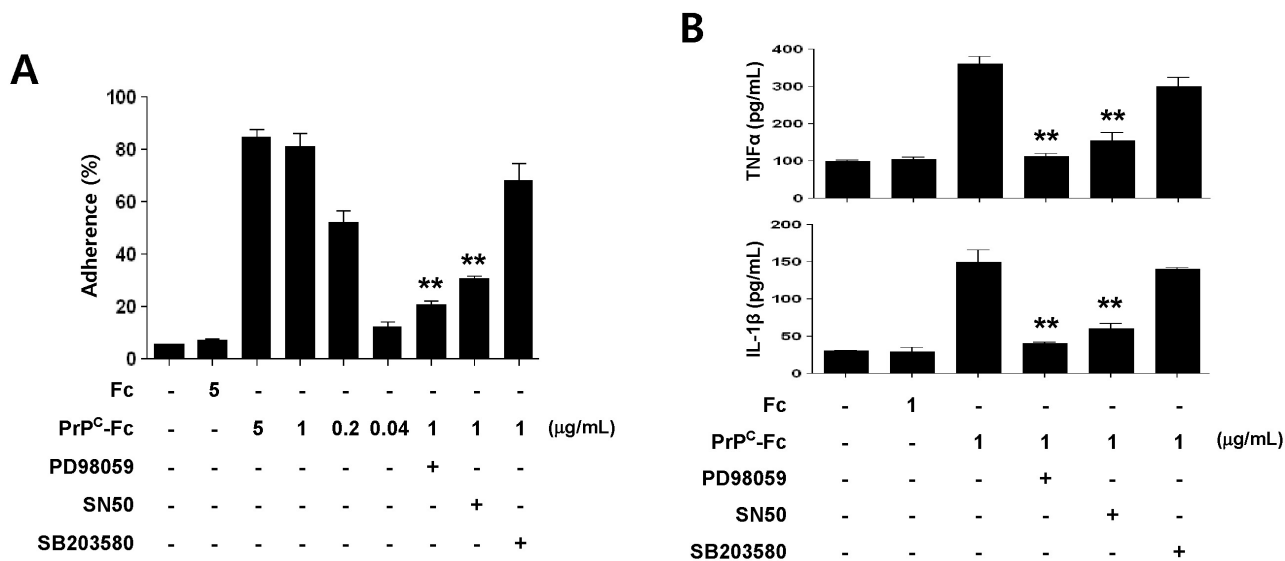


Figure 5. Role of ERK and NF- κ B signaling pathways in monocyte cell adherence and cytokine production induced by soluble PrP^C-Fc treatment. (A) THP-1 cells were treated with soluble PrP^C-Fc or control Fc at the indicated concentrations for 30 min, and adherent cells were counted. Specific signaling inhibitors such as PD98059 (20 μ M), SN50 (10 μ M), or SB203580 (10 μ M) were added to the THP-1 culture 1 h before soluble PrP^C-Fc treatment. Cell adherence is expressed as a percentage of the total number of cultured cells. The assay was performed in triplicate. Bar graphs represent the mean \pm SEM. Statistical analysis was performed in comparison with soluble PrP^C-Fc-treated cells. ****** p < 0.01. (B) Primary monocytes were treated with 1 μ g/ml soluble PrP^C-Fc or Fc control for 36 h. Specific signaling inhibitors such as PD98059 (20 μ M), SN50 (10 μ M), or SB203580 (10 μ M) were added to the monocyte culture 1 h before soluble PrP^C-Fc treatment. The concentrations of TNF- α and IL-1 β in the culture supernatant were determined with the Quantikine Assay Kit. The assay was performed in triplicate. Bar graphs represent the mean \pm SEM. Statistical analysis was performed in comparison with soluble PrP^C-Fc-treated cells. ****** p < 0.01. The data are representative of two independent experiments.

(21). Soluble murine PrP^C-Fc protein bound to the surface of mouse monocyte/macrophage cells, and activated Src-like kinases as well as Syk and Pyk2. In addition, ERK-1/2 and Akt were activated, suggesting a role for soluble PrP^C in phagocytosis, migration, and cytokine production by monocytes (21). In the current study, we also found activation of ERK and NF- κ B pathways in human monocytes in response to soluble PrP^C-Fc treatment. Furthermore, ERK and NF- κ B signaling pathways were found to be required for soluble PrP^C-Fc-induced adherence and cytokine production by monocytes. Further studies will be required to elucidate the precise role of each signaling pathway in the regulation of monocyte function by soluble PrP^C protein.

Although binding of soluble PrP^C to the cell surface of monocytes was demonstrated in the current study (Fig. 1B) and in a previous study (21), we did not identify a binding partner for soluble PrP^C on the surface of monocytes. PrP^C is known to bind to the neural cell adhesion molecule (N-CAM) (24), and to the laminin receptor and laminin receptor precursor protein (25-27). A putative role for a homotypic interaction between soluble PrP^C and membrane PrP^C

also needs to be investigated. All these possibilities need to be investigated in future studies.

Physiological roles of PrP^C in monocytes and macrophages have been studied in PrP^C knockout mice. Originally, PrP^C was proposed to act as a negative regulator of phagocytosis because macrophages from PrP^C-deficient mice showed higher phagocytic activity than those from wild-type mice (18). In contrast, recent studies with another line of PrP^C-deficient mice demonstrated that PrP^C enhanced phagocytic activity of macrophages (19,20). In addition, pseudopodium extension arrest and cell detachment were observed in macrophages from PrP^C-deficient mice (19). In the present study, we focused on the effects of the soluble form of PrP^C on monocytes and showed that soluble PrP^C protein enhanced cell adherence and phagocytic activity of monocytes. Our results are consistent with the more recent studies with PrP^C knockout mice (19,20), however, membrane-bound PrP^C and soluble PrP^C need to be considered separately when data from PrP^C knockout mice are interpreted.

In conclusion, we studied the function of soluble PrP^C protein in regulation of monocyte function using a recombinant

form of soluble human PrP^C fused with the Fc portion of IgG1. We found that soluble PrP^C-Fc enhanced adherence, phagocytosis, and cytokine production by monocytes via activation of the ERK and NF- κ B signaling pathways. Detailed mechanisms for the action of soluble PrP^C on monocytes will be further investigated in subsequent studies.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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