Journal of The Korea Convergence Society
Vol. 13. No. 1, pp. 101-107, 2022
e-ISSN 2713-6353
DOI : https://doi.org/10.15207/JKCS.2022.13.01.101

Anti-inflammatory activity of a short peptide designed for anti-cancer: a beneficial off-target effect of tertomotide

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항암백신 tertomotide의 항염활성 연구

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Abstract Tertomotide is a peptide vaccine developed for anti-cancer therapy. Since it has been found to ameliorate inflammatory symptoms in animal studies and clinical test, we investigated anti-inflammation activity of the tertomotide and the mechanism of action in monocyte in order to assess if tertomotide may serve as an anti-inflammatory agent by checking inflammatory cytokines and related signaling pathway following tertomotide treatment. We found that tertomotide reduced the level of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8 in LPS- or PMA-stimulated monocyte cell line and suppressed NF-κB signaling including the activation of ERK1/2 and P38 MAPK following TNF-α treatment. These results may correlate to the beneficial findings in animal studies, implicating that tertomotide may act as a potential anti-inflammatory agent. This study is an exemplary case for convergence that a computationally designed peptide for immunological purpose exerting unexpected biological activity may elicit novel anti-inflammatory drug.

Key Words : Convergence, Tertomotide, Anti-inflammation, Cytokine, Monocyte, NF-κB signaling

요 약 Tertomotide는 항암제로 개발된 펩타이드 백신이다. 그러나 동물실험과 임상시험에서 염증성 증상이 완화되는 현상이 발견된바 있다. 이에 tertomotide가 항염물질로 작용하는지 확인하기 위하여 직접적인 항염활성과 그 작용기전을 조사하였다. 이를 위해 LPS 또는 PMA에 의해 활성화된 monocyte에 tertomotide를 처리한 후 염증성 cytokine 생산과 관련된 신호전달과정을 관찰하였다. Monocyte에서 tertomotide는 TNF-α, IL-1β, IL-8 등 염증성 싸이토카인의 생산을 감소시켰고 NF-κB 신호를 감쇄시켰으며 또한 TNF-α에 의한 ERK1/2와 P38 MAPK의 활성화를 저해하였다. 이 결과는 tertomotide 처치에 따른 염증성 질환 완화가 NF-κB/STAT3의 신호의 감쇄와 항염활성에 의한 것이라고 설명할 수 있고 이를 활용하여 신규 항염 약물의 도출이 가능할 것으로 판단된다. 이는 면역학적 활성을 목표로 계산화학적으로 설계된 물질의 생물학적 성질을 활용하여 새로운 약물을 도출하는 융합연구의 예시가 될 것이다.

주제어 : 융합, Tertomotide, 항염활성, 싸이토카인, 단핵구(monocyte), NF-κB 신호전달

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Received December 5, 2021 Revised December 22, 2021
Accepted January 20, 2022 Published January 28, 2022

1. Introduction

1.1 Anti-cancer vaccine, tertomotide

Tertomotide is a 16 amino acid-long peptide derived from human telomerase reverse transcriptase (hTERT) [1,2]. Tertomotide was originally developed as a therapeutic vaccine to treat various cancers such as pancreatic cancer, melanoma, non-small cell lung cancer, etc. Tertomotide is derived from hTERT and designed to bind to multiple MHC (Major Histocompatibility Complex) class II and MHC class I molecules. It has been shown that tertomotide have immunogenic tendencies activating $CD4^+$ (cluster of differentiation 4 positive) and $CD8^+$ (cluster of differentiation 8 positive) T cells [1,3]. Meanwhile, this short peptide exerted an interesting off-target effect ameliorating symptoms related to inflammation in clinical studies and in animal studies [2], suggesting that tertomotide may serve as an anti-inflammatory agent.

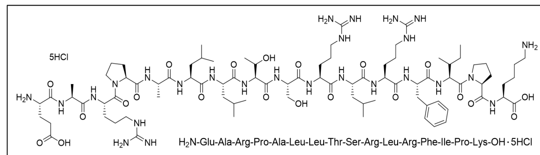

Fig. 1. Molecular structure of tertomotide hydrochloride

1.2 Anti-inflammatory activity of tertomotide

Since tertomotide has been computationally designed to be an immunogen to deliver the information of the peptide sequence to human immune system (Fig. 1), the anti-inflammatory property is quiet unexpected. Recent studies have demonstrated that tertomotide suppresses inflammatory response in cultured human dental pulp cells and in primary peripheral blood mononuclear cells [4,5]. The anti-inflammatory effects of tertomotide have been investigated C57BL6/J mice after bilateral ischemia-reperfusion injury (IRI), in laser-induced choroidal neovascularization rat model and in hepatitis model implying tertomotide definitely correlates to the inflammatory symptoms [6-8]. In this regard, we investigated the effects of tertomotide on inflammatory response in a cultured monocyte cell line, which is regarded to be the first cell to encounter the tertomotide upon administration.

In order to evaluate the anti-inflammatory effects of tertomotide, PMA/LPS-induced THP-1 cell model was exploited as THP-1 is a human monocytic leukemia cell line which differentiates into macrophage -like cells in response to the treatment of phorbol esters such as PMA and increases the inflammatory activity induced by LPS [9].

Inflammation is known to be closely related cancer progression. Both the tumor and the normal tissue produce pro-inflammation cytokines which cause a complex cascade of biological responses leading to cachexia [11,12]. However, the cancer-related inflammation is not supposed to be directly affected, since the administration route of tertomotide as a anti-cancer vaccine is intradermal injection. Nonetheless, the anti-inflammatory effect of tertomotide has been intensively investigated in various disease models due to the findings in clinical studies over decade highlighting the potency of tertomotide as a anti-inflammatory agent. In this context, we investigated the anti-inflammatory effects of tertomotide in TNF-α induced cancer cell lines.

2. Materials & methods

2.1 Quantitative RT-PCR measurements

Total RNAs were extracted and purified by RNeasy Mini Kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized from total RNAs using the Reverse Transcription System

(Promega, Madison, Winsconsin, USA). To determine the relationship between cycle number (C_t) and mRNA levels, primers were calibrated using serial dilutions of cDNA and genomic DNA. Quantitative RT-PCRs were performed with an CFX96 Real-Time System (Bio-Rad) using the RT^2 SYBR Green qPCR Mastermix kit (Qiagen) as described by the manufacturer (Qiagen) in a total volume of 25 μL. The relative expressions were analyzed by CFX Manager™ Software V3.0 using the $\Delta\Delta C_t$ method, and an average of the expression of the reference gene, *β-actin*, was used as control for template levels. Each experiment was performed in triplicate.

2.2 Cell viability assay

To evaluate the effect of tertomotide on cell growth, cell viability was analyzed using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells were seeded in 96-well plate (2 ~ 5 × 10^3 cells/well) overnight. Then, the cells were incubated with varying concentration of tertomotide (0.001 ~ 100 μM). After incubation for the indicated time, 10 μL of kit reagent, to each well was added WST-8 (water-soluble tetrazolium-8) and the plate was incubated for 2 hours at 37 °C. The absorbance at 450 nm was measured using Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

2.3 Cytokines secretion assay

Cytokines were measured in supernatant using ELISA method as described by the manufacturer (R&D Systems, Minneapolis, MN, USA). The Quantikine Immunoassay Human IL-1β and SixPak Human TNF-α kits were used (R&D Systems). Finally, wells were measured and analyzed at 450 nm using Synergy 2 Multi-Mode Microplate Reader (BioTek).

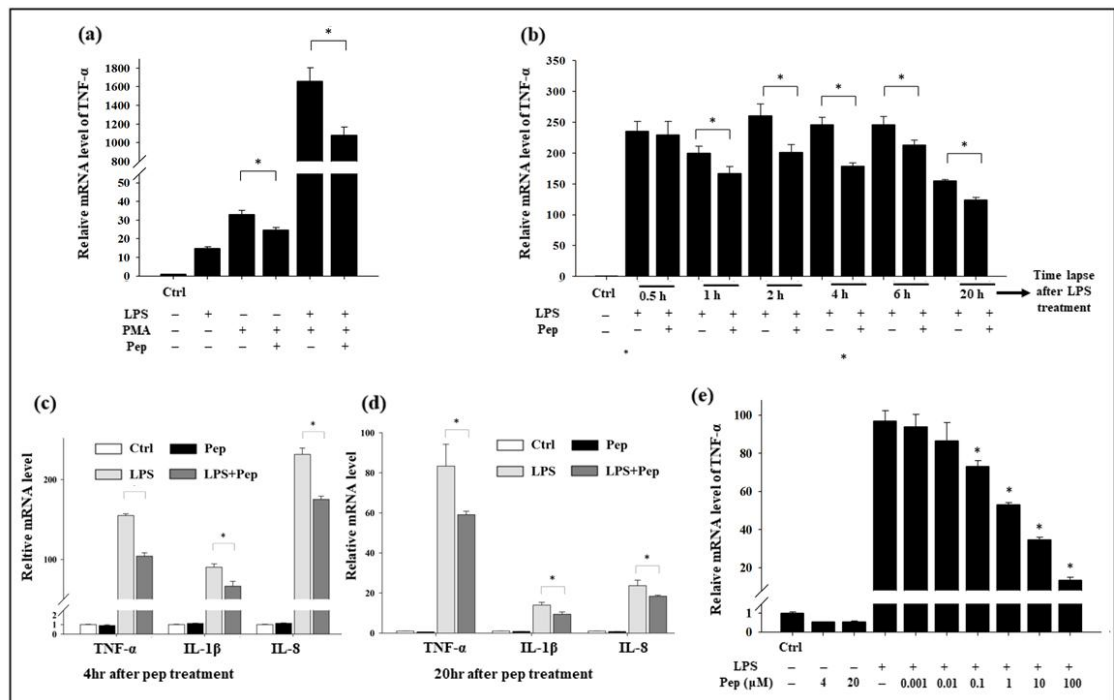

Fig. 2. Tertomotide (Pep) reduces mRNA levels of pro-inflammatory cytokines in activated THP-1 cells.

2.4 NF-κB Nuclear translocalization studies by confocal microscopy

THP-1 cells (ATCC, Manassas, VA, USA) were seeded in 8-well Lab-Tek II Chambers (NUNC, ThermoFisher Scientific, Waltham, MA, USA) and cultured to 70% confluence. After stimulation for 20 hours with 100 ng/mL PMA (Phorbol-12-myristate-13-acetate, Sigmaaldirich, Burlington, MA, USA), cells were pre-treated with 10 nM tertomotide for 2 hours. Following treatment with 50 ng/mL of Recombinant Human TNF-α (R&D systems) for 20 minutes or 1 μg/μL LPS (lipo-polysaccharides, Sigmaaldirich, Burlington, MA, USA) for 30 minutes, the cells were treated with cold Phosphate-buffered saline (PBS) for 1 minute, then fixed in 4% cold formaldehyde in PBS for 5 minutes [13]. After fixation, the cells were blocked and permeabilized in 5% normal goat serum (with 0.1 % Triton X-100) for 2 hours RT, and then incubated with anti-NF-κB (p65, phosphorylated P65 or p50) antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4 °C, followed by washing and incubation with Alexa Flour 647 goat anti-mouse IgG (H+L) (invitrogen, Carlsbad, CA, USA) or Alexa Fluor 555 goat anti-rabbit IgG (H+L) (invitrogen) for 1 hour at room temperature. Finally, after washing 4 times with PBS, specimens were mounted and stained on coverslips by SlowFade® Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen). Images were taken by using Olympus FV1000 Confocal Microscopy (Olympus, Tokyo, Japan).

2.5 Transcription Factors Activity Analyses by Western Blotting

2.0×10^6 THP-1 cells were seeded in 100 mm dish then stimulated for 20 hours with 100 ng/mL PMA. THP-1 cells were pre-treated with 0 ~ 10 μM tertomotide for 2 hours [13]. Following the treatment with 50 ng/mL of Recombinant Human TNF-α for 20 minutes, the dishes were washed with ice-cold PBS twice and were frozen in liquid nitrogen immediately. The frozen cells were lysed in RPIA lysis buffer (ThermoFisher Scientific) which contains Complete Mini EDTA-free Protease Inhibitor and phosphatase inhibitor Cocktail Tablets (Roche). The lysates were clarified at 4 °C by centrifugation at 12,000 rpm for 5 minutes, and the protein concentrations were determined using Bio-Rad Protein Assay Reagent (Bio-Rad, Munich, Germany). 20 μg per well protein were first loaded in Novex 4-20% Tris-Glycine Gel (invitrogen) and separated by XCell SureLock Mini-Cell (invitrogen) and then transferred to iBlot Gel Transfer Stacks PVDF membrane (invitrogen) by iBlot Dry Blotting System (Invitrogen). The membranes were blocked with 5% milk in TBST for 1 hour, incubated overnight with primary antibodies (all primary antibodies were purchased from Cell Signaling Technology, Danvers, MA, USA), and then probed for 1 hour with secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Invitrogen). After extensive washing with Tris-buffered saline with Tween 20 (TBST, Sigmaaldirich), the target proteins were detected on the membranes by ECL™ Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL, USA). Images were taken by using Gel Logic 4000 Pro (Carestream, Rochester, NY, USA).

2.6 Statistical analysis

Data represent the mean ± Standard Deviation at least three independent experiments. Statistical significance was determined by Student's t-test. The P value less than 0.05 was deemed to be statistically significant, and labeled as "*".

3. Result

3.1 Tertomotide reduces mRNA levels of pro-inflammatory cytokines in LPS-induced THP-1 cells

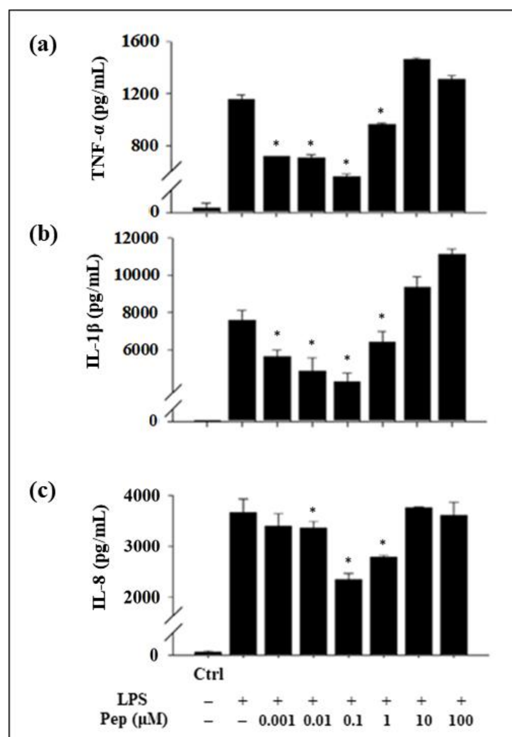

Fig. 3. Tertomotide (Pep) suppresses secretion of proinflammatory cytokines in LPS induced THP-1 cell lines.

3.2 Tertomotide suppresses the secretion of pro-inflammatory cytokines

To examine the anti-inflammatory effect of tertomotide on protein levels, the secretion of pro-inflammatory cytokines were measured via ELISA. For macrophage model, THP-1 cells were stimulated by 100 ng/mL PMA for 20 hours, and then co-treated with 10 ng/mL LPS and tertomotide of varying concentration [13]. ELISA was performed after 4 hours. Data shows that 1 nM tertomotide is sufficient to significantly suppress TNF-α secretion whereas 0.1 μM tertomotide exhibits the biggest inhibition rate. Reversely, secretion of TNF-α was increased over the dose of 10 μM tertomotide (Figure 3(a)). This pattern was observed in IL-1β and IL-8's ELISA data as well (Figure 3(b) and (c)).

3.3 Tertomotide Does not Affect Proliferation and Viability of THP-1 Cell Lines

Tertomotide was treated in a various concentration (1 nM ~ 100 μM) to determine the cytotoxicity. There is no noticeable effect on THP-1 cell lines even with 100 μM tertomotide within 5 days incubation (data not shown).

3.4 Tertomotide inhibits nuclear translocation of NF-κB complex

Tertomotide appeared to inhibit the nuclear translocation of NF-κB P65 in confocal studies. In these tests, NF-κB activity was induced by two independent activators, TNF-α and LPS to cross confirm the result. Since the cytokine level of appeared to be increased at higher concentrations, 10 nM of tertomotide was adopted for this test to rule out the possibility that locally high concentration of tertomotide might interfere with normal cellular response. Results show that 10 nM tertomotide (Pep) would be sufficient to suppress nuclear translocation of phosphorylated P65 which was induced by either TNF-α or LPS. Furthermore, the signal of both P65 and phosphorylated P65 in TNF-α or LPS stimulated cells was stronger than that in tertomotide co-treated cells (Figure 4(a), (b)).

3.5 Tertomotide Inhibits NF-κB, P38 MPAK and ERK signaling pathways

The phosphorylation of NF-κB P65 was demonstrated to be reduced by 10 nM tertomotide (Pep) treatment (Figure 4(a), (b)). To confirm this result, Western Blot analysis was performed in the presence of tertomotide in various concentrations. Besides, we investigated

whether or not other signaling molecules are also affected by tertomotide treatment. Thus, P38 MAPK (mitogen-activated protein kinase) and extracellular signal-regulated kinase (ERK 1/2) were also tested in the same manner. Tertomotide turned out to suppress the phosphorylations of NF-κB P65, P105 (P50), P38 MAPK and ERK 1/2 (Figure 5). The phosphorylations of NF-κB P65, P105 (P50) were suppressed in a dose dependent manner up to the concentration of 100 nM, while the phosphorylation of P38 MAPK was suppressed at the concentration range between 1 nM and 1 μM. In addition, the phosphorylation of ERK 1/2 appeared to be suppressed in a dose dependent manner in tested concentrations.

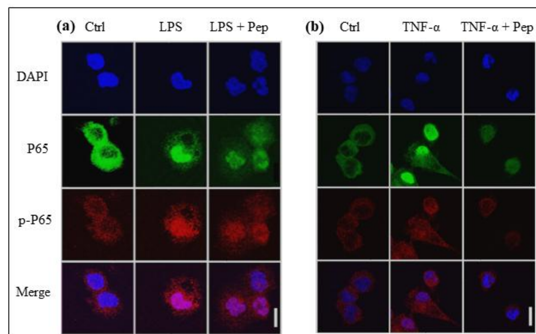

Fig. 4. Tertomotide (Pep) inhibits nuclear translocation of both P65 and phosphorylated P65 in LPS or TNF-α stimulated THP-1 cells.

4. Discussion

Tertomotide has been originally developed as a therapeutic vaccine peptide based on hTERT, and undergone clinical test to treat various cancers [1-3,14]. Hence, this peptide has been validated as immunogenic material which delivers the information on the peptidyl sequence to immune system. However, the unexpected off-target effect on inflammatory symptoms prompted us to further investigation.

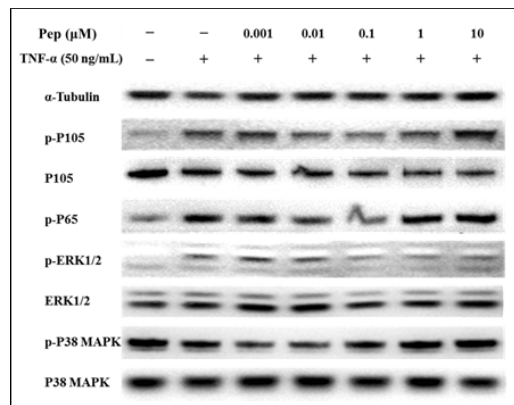

Fig. 5. Tertomotide (Pep) inhibits phosphorylation of NF-κB, P38 MPAK and ERK on THP-1 cells.

In this study, tertomotide demonstrated to inhibits the production of pro-inflammation cytokines in activated monocyte cell line (THP-1). Next, we investigated the impact on NF-κB signaling as NF-κB is a pivotal regulator in inflammation in most types of cells. Tertomotide exerted a suppressive effect on NF-κB signaling pathway. The confocal study demonstrated that tertomotide dramatically reduced the nuclear translocalization of P65, which is a critical event for signaling as concomitantly found in previous studies by others [4-8]. Tertomotide consequently turned out to reduce the production of inflammatory cytokine via reducing the phosphorylation of NF-κB P105 and P65. The tertomotide-mediated anti-inflammatory activity is demonstrated to be associated with reduced phosphorylation of MAPKs including ERK1/2 and p38 MAPK. Since this signaling is responsible for blocking the formation of reactive oxygen species, the effect of tertomotide appears to be exerted in a similar route as it is known to be a common action for anti-inflammatory agents as supported by previous report [15].

Taken together, we concluded that the attenuation of inflammatory symptoms by

tertomotide may be achieved via blocking NF-κB signaling resulting in reductions in related cytokines. However, peptides tends to aggregate at high concentration causing adverse effects due to the amphiphillic nature [16]. Arbeit that the detailed mechanism of action at molecular level is still unclear and the dose control is tricky, it is suggested to subject tertomotide to therapeutic applications.

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