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Original Article

Effects of L-ascorbic acid on the production of pro-inflammatory and anti-inflammatory cytokines in C57BL/6 mouse splenocytes

Eun Hee Kong,[1] Sun Young Ma,[2] Jee Yeong Jeong,[3*] Kwang Hyuk Kim[4*]

[1]Department of Family Medicine, Kosin University College of Medicine, Busan, Korea
[2]Department of Radiation oncology, Kosin University College of Medicine, Busan, Korea
[3]Department of Biochemistry, Kosin University College of Medicine, Busan, Korea*
[4]Department of Microbiology, Kosin University College of Medicine, Busan, Korea*

쥐의 비장세포에서 아스코르빈산의 사이토카인에 대한 영향

공은희,[1] 마선영,[2] 정지영,[3*] 김광혁[4*]

[1]고신대학교 의과대학 가정의학과 교실
[2]고신대학교 의과대학 방사선종양학과 교실
[3*]고신대학교 의과대학 생화학 교실
[4*]고신대학교 의과대학 미생물학 교실

Objective: The imbalance between pro-inflammatory and anti-inflammatory cytokines may underlie different pain states. Although ascorbic acid is the most important physiological antioxidant that affects host defense mechanisms and immune homeostasis, there is limited information on the effects of ascorbic acid on the production of cytokines.

Methods: In this study, we investigated the in vitro effect of L-ascorbic acid (AA) on the production of pro-inflammatory and anti-inflammatory cytokines by stimulating C57BL/6 mouse splenocytes with the polyclonal activators lipopolysaccharide or concanavalin A.

Results: AA significantly downregulated the expression of IL-6, IL-12, and TNF-α at 48 h and 72 h in mouse splenocytes treated with a combination of polyclonal activators and AA. AA treatment also resulted in upregulation of IL-4 and IL-10 at 72 h. These findings demonstrated that AA significantly potentiated production of anti-inflammatory cytokines whereas there was an inverse association between AA and expression of pro-inflammatory cytokines in mouse splenocytes.

Conclusion: AA may have potential applications in the reduction of inflammatory pain because of its function in modulating the production of cytokines. However, further in vivo investigations are necessary to elucidate the mechanisms involved.

Key Words: Ascorbic acid, Interleukin, Tumor necrosis factor

Corresponding Author : Jee yeong Jeong, Department of Biochemistry, Kosin University College of Medicine 262, Gamcheon-ro, Seo-gu, Busan, South Korea (602-703)
TEL: +82-51-990-6365 FAX: +82-51-990-3045 E-mail: fmeunhee@gmail.com

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Inflammatory stimuli or tissue injuries induce inflammatory pain through the release of cytokines. Pro-inflammatory cytokines generate inflammatory pain[1] by reducing thermal or mechanical pain thresholds.[1-2] During the prolonged action of pro-inflammatory cytokines, anti-inflammatory cytokines are released in stages to inhibit the production and action of the pro-inflammatory cytokines.[1] Antagonists of pro-inflammatory cytokine may reduce hyperalgesia in inflammation models. Several micronutrients, such as vitamins C, E, and D, have recognized antioxidant and anti-inflammatory properties. Vitamin C is the most important physiological antioxidant that affects host defense mechanisms and immune homeostasis.[3-5] It modulate the host susceptibility or resistance to the infections caused by bacteria, viruses or parasites.[4] Delayed-type hypersensitivity skin test, an index of immune response, is reduced in healthy men with the vitamin C intakes.[3] The supplementation of vitamin C increases serum levels of antibodies in humoral immunity.[5]

Lipopolysaccharide (LPS) or concanavalin A (Con A)-induced splenocytes of 8-week-old C57BL/6male mice have proliferated by L-ascorbic acid (AA) in vitro.[6] Splenocytes produce pro-inflammatory cytokines TNF-α, IL-6 and anti-inflammatory cytokine IL-10 by the inflammatory stimuli or tissue injuries.[7] However, there is limited information on the effects of vitamin C on the production of cytokines. The aim of the present study was to study the *vitro* effect of L-ascorbic acid (AA) on the production of pro-inflammatory and anti-inflammatory cytokines by stimulating C57BL/6 mouse splenocytes with polyclonal activators such as LPS or Con A.

Materials and Methods

Animals

This study (IRB No. Kosin 13-04) was approved by an institutional animal care and use committee of the Kosin University College of Medicine, Busan, Korea, in accordance with approved published guidelines, prior to performing the research and publishing the data.

Male 6-week-old C57BL/6 mice (B.W 25g) were purchased from Daehan BioLink (Eumseong-gun, Chungcheongbuk-do, Republic of Korea) and housed individually in cages. Spleen cells were suspended in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) with 10% bovine serum. The cells were plated in 24-well culture dishes (Costar, Cambridge, MA, USA) at a concentration of 2×10^6 cells per well. Non-adherent cells were removed by aspiration and the wells were washed three times with PBS. The adherent cells were cultured for 24 hours in RPMI 1640 media with 10% bovine serum.

Cytokine assays

Disturbances in cytokine synthesis can be best studied under dynamic conditions by stimulating competent cells with polyclonal activators such as LPS or Con A and analyzing the patterns of cytokine production.[8] LPS (2 μg/ml, Sigma-Aldrich Corp., USA) or Con A (2 μg/ml, Pharmacia Chemicals, Sweden) were added to the splenocytes as stimulators prior to incubation with low dose 10 μg/ml[9-10] 176.12 M.W. AA (Sigma-Aldrich Corp., USA) for 6, 24, 48, or 72 h at 37℃ in a humidified atmosphere containing 5% CO_2. No toxicity was observed within the concentration range used in this study. Splenocytes were treated with dimethyl sulfoxide (DMSO) (0.01%, Gibco BRL) as a control.

At the end of the culture period (6, 24, 48, or 72 h) the supernatants were harvested and centrifuged at 300 × g for 10 minutes, followed by a second centrifugation at 1000 × g for 30 minutes. The supernatants of mouse splenocytes exposed to cell stimulants (LPS, Con A) alone or to AA plus LPS or Con A were stored at -70℃ prior to assaying for cytokines. Cytokine concentrations were measured using enzyme-linked immunosorbant assay (ELISA) kits for mouse tumor necrosis factor-α (TNF-α), interleukin (IL)-1ß, IL-2, IL-4, IL-6, IL-10, and IL-12 (Biolegend, San Diego, CA, USA). The 96-well plates were coated with commercial capture antibodies at a concentration of 100 μl/well by incubation at 4℃ overnight. After washing (four washes for 1 min each), 200 μl of assay diluent was added to each well and incubated for 1 h at room temperature. The plates were washed (four washes for 1 min each), and 100 μl of the sample was added to the wells and incubated for 2 h at room temperature. After washing (four washes for 1 min), 100 μl of detection antibody for the appropriate cytokine was added to each well, and the plates were incubated for 1 h at room temperature. After further washing (four washes for 1 min), 100 μl of avidin-horseradish peroxidase was added to each well, and plates were incubated for 30 min at room temperature. After a final wash (five washes for 1 min), 100 μl of the substrate solution mixed with tetramethylbenzidine (Abcam Inc, Cambridge, MA) was added for color development for 20 min at room temperature. The reaction was stopped by addition of 100 μl stop solution. Absorbance was measured at 450 nm on an ELISA microplate reader (model 550 microplate reader, Bio-Rad, Richmond, VA, USA).

Statistical analysis

Data were analyzed by Student's *t*-test. Results are presented as means or means ± SD. Differences were considered statistically significant at $P < 0.05$.

Results

1. Effects of AA on the production of pro-inflammatory cytokines in mouse splenocytes

The expression of IL-6 and TNF-α was inhibited significantly at 48 h and 72 h in mice splenocytes treated with Con A plus AA (Fig. 1A and 1B). In addition, treatment of splenocytes with LPS plus AA significantly downregulated the expression of IL-12 at 48 h and 72 h (Fig. 1C). However, co-administration of AA with stimulants (LPS or Con A) did not significantly affect the expression of IL-1ß and IL-2 (data not shown).

2. Effects of AA on the production of anti-inflammatory cytokines in mouse splenocytes

Co-administration of Con A and AA to mouse splenocytes resulted in upregulation of IL-4 and IL-10 at 72 h compared with treatment with Con A alone, whereas co-administration of LPS and AA did not significantly affect the expression of IL-4 and IL-10 (Fig. 2A and 2B).

Discussion

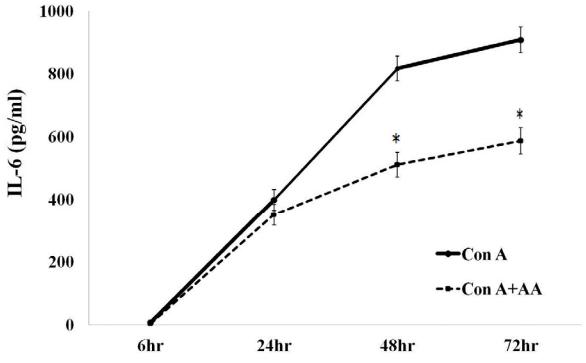
In the present study, AA significantly potentiated the production of anti-inflammatory cytokines such as IL-4 and IL-10 in mouse splenocytes. We also found an inverse association between AA treatment and the production of pro-inflammatory cytokines IL-6, IL-12, and TNF-α.

Some studies have shown that tissue levels of cytokines correlate with hyperalgesia in a number of painful states.[11] Cytokines are the most important links between the immune system and nociception.[12] In animal models pro-inflammatory mediators excite nociceptors and cause hypersensitivity,[12] and the balance between pro- and anti-inflammatory cytokines modulates pain sensitivity.[13] Although cytokines have well-described roles in inflammatory pain, the regulation of their production and release by AA is not well understood. This study showed inverse associations between AA and the pro-inflammatory cytokines IL-6, IL-12, and TNF-α. However, several intervention trials reported no association between circulating AA and various inflammatory biomarkers.[14-15]

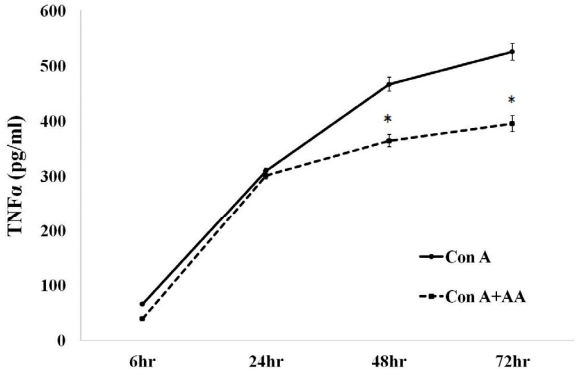
Pro-inflammatory cytokines such as TNF-α induce and facilitate neuropathic and inflammatory pain.11,[16-17] TNF-α initiates the activation cascade of cytokines, chemokines, and growth factors involved in the inflammatory response and therefore is generally accepted as the prototypic pro-inflammatory cytokine.[12] Consistent with this, TNF-α is synthesized and released in tumor tissue and induces heat hypersensitivity

Figure and Figure Legend

1A



1B



1C

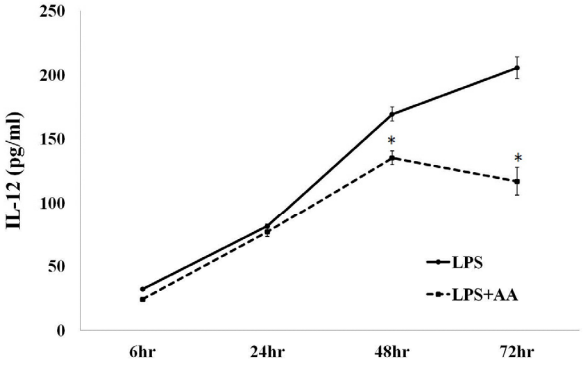


Fig. 1. Effects of L-ascorbic acid on the production of pro-inflammatory cytokines in mouse splenocytes

The expression of IL-6 and TNF-α was inhibited significantly at 48 h and 72 h in cells treated with Con A plus AA (Fig. 1A and 1B). In addition, treatment of splenocytes with LPS plus AA significantly downregulated the expression of IL-12 at 48 h and 72 h (Fig. 1C). $^{*}P<0.05$ by Student's t-test.

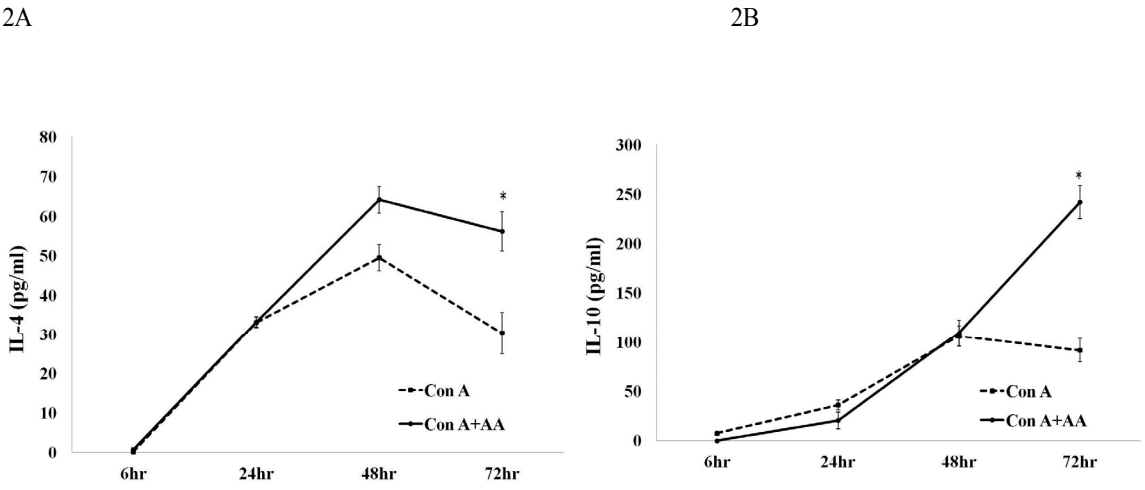


Fig. 2. Effects of L-ascorbic acid on the production of anti-inflammatory cytokines in mouse splenocytes

Co-administration of Con A and AA resulted in upregulation of IL-4 and IL-10 at 72 h compared to treatment with Con A alone, whereas co-administration of LPS and AA did not significantly affect the expression of IL-4 and IL-10 (Fig. 2A and 2B). $^{*}P<0.05$ by Student's t-test.

and pain by directly affecting nociceptors innervating the tumor area. Inflammation induces hypernociception that is mediated by an initial release of TNF-α, which in turn triggers the subsequent release of IL-1ß.[18] IL-1ß is the most-studied member of the expanding IL-1 family because of its role in mediating auto-inflammatory diseases.[19] However, in the present study the levels of IL-1ß in culture supernatants of mouse splenocytes exposed to AA were not significantly different from those of mice exposed to LPS or Con A alone. The classic pro-inflammatory interleukin IL-6 is an important neuronal survival and neurite elongation factor,[20-21] whose functions involve nociceptors that express signal transducer components at the cell membrane.[22-23] IL-6 is produced and excreted by immune cells including macrophages, glia cells, and even neurons.[24] Increased levels of IL-6 have been correlated with sickness behavior in humans[25] and treatment-associated symptoms including pain and fatigue.[26] In addition to controlling immune cell interactions, IL-6 may also be responsible for the pain and hypersensitivity associated with inflammation, neuropathy, or cancer by directly regulating the gain of pain-sensing neurons.

On the other hand, the anti-inflammatory cy-

tokines IL-4 and IL-10, which can suppress pro-inflammatory cytokine production, exhibit antinociceptive effects in different pain models.[27-28] We found that AA upregulated the expression of IL-4 and IL-10 in mouse splenocytes. Considering that TNF-α and IL-1ß contribute to the generation of inflammatory pain and IL-4 and IL-10 have anti-hyperalgesic effects, it is conceivable that AA induces both attenuation of the increase in TNF-α and IL-1ß and augmentation of the increase in IL-4 and IL-10. However, we did not prove the dose dependent effect of AA on the anti-inflammatory or anti-inflammatory cytokines.

Severe pain persists in many patients even with high-dose analgesic therapies. Individual variations in the severity of pain and in the responsiveness to treatment have been assumed to result from sociodemographic characteristics (age, sex, race, marital status), clinical health status (nutritional status, performance status, comorbid conditions), or disease-related variables (stage of disease). Cytokines are strongly linked to inflammation, neuropathy, and cancer. There is increasing evidence that the balance between proalgesic and antialgesic cytokines is related to the severity and persistence of the accompanying pain.[29-30] However, further in vivo investigations of this process are necessary to elucidate the mechanisms involved.

Conclusion

Our results demonstrate that AA induced significant downregulation of IL-6, IL-12, and TNF-α expression in mouse splenocytes, whereas the expression of IL-4 and IL-10 was significantly increased when Con A was used as a stimulant. On the basis of these results, we suggest that AA elicits attenuation of inflammatory pain by reducing the expression of pro-inflammatory cytokines and increasing the expression of anti-inflammatory cytokines. Therefore, compared with treatment with a cellular stimulant (LPS or Con A) alone, co-administration of AA and cellular stimulants to suspensions of mouse splenocytes resulted in downregulation of L-6, IL-12, and TNF-α, and upregulation of IL-4 and IL-10. Through this mechanism, AA may have potential therapeutic applications in inflammatory pain.

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