Article

Direct Effects of Activin A on the Activation of Mouse Macrophage RAW264.7 Cells

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Macrophages play critical roles in innate immune and acquired immune *via* secreting pro-inflammatory mediators, phagocytosing microorganisms and presenting antigens. Activin A, a member of transforming growth factor β (TGF-β) superfamily, is produced by macrophages and microglia cells. In this study, we reported a direct effect of activin A as a pro-inflammatory factor on mouse macrophage cell line RAW264.7 cells. Our data revealed that activin A could not only increase IL-1β and IL-6 production from RAW264.7 cells, but also promote pinocytic and phagocytic activities of RAW264.7 cells. In addition, activin A obviously up-regulated MHC II expression on the surface of RAW264.7 cells, whereas did not influence MHC I expression. Activin A also enhanced CD80 expression, which is a marker of activated macrophages, but did not influence RAW264.7 cell proliferation. These data suggest that activin A may regulate primary macrophage-mediated innate and acquired immune response *via* promoting the activation of rest macrophages. *Cellular & Molecular Immunology*. 2009;6(2):129-133.

Key Words: activin, macrophage, MHC II, CD80

Introduction

Macrophages have an important role in innate immune defense against microbial infections, which involves secretion of pro-inflammatory mediators and phagocytic activities (1, 2). Macrophages also play a critical role in acquired immune response *via* their antigen-presenting activities. Activin A, a multifunctional factor of the transforming growth factor-beta (TGF-β) superfamily, is mainly produced by microglia and macrophages, and both its anti-inflammatory and pro-inflammatory activities are related to macrophage functions (3-6). As an anti-inflammatory factor, activin A is involved in regulation of acute phase response in inflammatory diseases *via* autocrine and paracrine manner, and also acts as an antagonist of interleukin (IL)-6 and IL-11, which can be produced by macrophages (5-7). Recently our studies have demonstrated that activin A could down-regulate the phagocytosis of lipopolysaccharide (LPS)-activated mouse peritoneal macrophages *in vitro* and *in vivo*, which confirms the anti-inflammatory role of activin A (1). However, activin A is also a pro-inflammatory factor and increases dramatically in inflammatory situations. Lots of experiments have demonstrated that activin A is involved in the pathogenesis of fibrotic human diseases and induction of extracellular matrix expression in hepatocytes (8-10). Our previous studies have found that activin A can increase the production of inflammatory mediator, nitric oxide (NO) in mouse peritoneal macrophages (3). However, the direct effects of activin A on phagocytic and antigen-presenting activities of rest macrophages still remain unclear.

In the present study, we examined the production of IL-1β and IL-6, as well as the phagocytosis and pinocytosis of mouse macrophage cell line RAW264.7 cells induced by activin A. Simultaneously we also analyzed MHC I and MHC II expressions, which are involved in the presentation of endogenous and exogenous antigens, respectively, as well as the expression of CD80, an activation marker of macrophages. These studies will help us to understand direct

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Abbreviations: TGF-β, transforming growth factor β; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

effects of activin A as a pro-inflammatory factor on the activation of rest macrophage RAW264.7 cells.

Materials and Methods

Reagents and antibodies

Human activin A (Act) was obtained from R&D (Minneapolis, MN 55413); MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was purchased from Sigma (St. Louis, MO, USA); IL-1β and IL-6 enzyme-linked immunosorbent assay (ELISA) kits and mouse monoclonal antibodies against surface molecules (CD80) were obtained from BD Biosciences (San Jose, CA, USA); fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against MHC I and MHC II were purchased from AbD Secrotec (Oxford, OX5 1GE, UK).

Cell culture

RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in a humidified incubator containing 5% CO_2 at 37°C in 10% fetal calf serum (FCS)/RPMI 1640 medium. Cells were seeded in 96-well plates at a density of 1×10^5 cells/well and incubated in 5% FCS/RPMI 1640 medium in the presence or absence of activin A (0.8-10 ng/ml) for 24 h, respectively.

IL-1β and IL-6 detection by ELISA

IL-1β and IL-6 levels in the supernatant of cultured cells were measured by using commercial two-site mouse IL-1β and IL-6 ELISA kits according to the manufacturer's protocol, respectively. The absorbance was then detected at 450 nm to evaluate IL-1β and IL-6 levels.

Pinocytosis assay of RAW264.7 cells

RAW264.7 cells plated to 96-well culture plates were incubated in 5% FCS/RPMI 1640 medium in the presence or absence of activin A (0.8-10 ng/ml) for 24 h, at 37°C in a humidified 5% CO_2 and 95% air incubator. Culture media were removed and 200 μl/well of 0.7% neutral red was added. Media were discarded after incubation for 1 h. The macrophages were washed twice with pH 7.4, 0.01 mol/L phosphate-buffered saline (PBS) and then lysed in 200 μl of lysis solution (1:1 of 0.1 mol/L acetic acid and 100% ethanol) at 4°C overnight. Absorbance was measured at 490 nm (11).

Phagocytosis assay of RAW264.7 cells

To evaluate phagocytic capability of macrophages, chicken red blood cells (cRBC) were used as antigen particles. RAW264.7 cells in 12-well plates were treated with or without activin A (2 ng/ml or 5 ng/ml) in 5% FCS/RPMI 1640 medium at 37°C in a humidified 5% CO_2 and 95% air incubator for 24 h, respectively. Then 1% cRBC were added and incubated for 1 h. Macrophages were rinsed with PBS and fixed with 4% paraformaldehyde. Cells were stained with Wright-Giemsa dye for 3 min and rinsed with PBS. Phagocytosed cRBC were examined with light microscopy and a minimum of 200 macrophages were counted in each well. The phagocytosis ratio (PR) and index (PI) of macrophages were calculated as follows: PR = number of macrophages phagocytosing cRBC/number of macrophages; PI = number of cRBC phagocytosed by macrophages/number of macrophages (12).

Flow cytometric analysis

The expressions of MHC I, MHC II and CD80 on the surface of RAW264.7 cells were analyzed by flow cytometry using anti-mouse MHC I, MHC II and CD80 antibodies, respectively. RAW264.7 cells were incubated with IgG at 4°C for 30 min to block Fc-receptor. The cells were washed twice with cold buffer and then incubated with FITC-conjugated anti-mouse CD80, MHC I and MHC II antibodies or FITC-conjugated IgG as isotype control for 30 min at 4°C, respectively. The cells were analyzed with FACSort Vantage (BD, Franklin Lakes, NJ). The data were collected and analyzed with CellQuest software (BD Biosciences) to assess the percentage of fluorescence positive cells.

Proliferation analysis of RAW264.7 cells

RAW264.7 cells were seeded into 96-well plates at a density of 2×10^5 cells/ml, and incubated in 200 μl of 5% FCS/RPMI 1640 medium in the presence or absence of activin A (0.8-10 ng/ml) at 37°C for 24 h in a humidified 5% CO_2 and 95% air incubator. The viable cells were stained with MTT for 4 h. Media were removed and the formazan crystala was dissolved by adding 200 μl of dimethylsulfoxide (DMSO). Absorbance was detected at 570 nm to express the cell viabilities (13).

Statistical analysis

The data were expressed as means ± SD and statistic analysis was performed by Student's t test. $p < 0.05$ was considered statistic significance.

Results

Activin A induces IL-1β and IL-6 production in RAW264.7 cells

To investigate the possible function of activin A as a pro-inflammatory factor, effects of activin A on the induction of inflammatory cytokines, IL-1β and IL-6 in RAW264.7 cells were first examined. We found that activin A significantly induced IL-1β and IL-6 production in RAW264.7 cells in a dose-dependent manner (Figure 1).

Activin A promotes both phagocytosis and pinocytosis of RAW264.7 cells

Both phagocytic and pinocytic activities are the most important functions of macrophages in innate immune response. To assess effects of activin A on macrophage functions, the phagocytic and pinocytic activities of macrophages to cRBC and neutral red were examined respectively. As shown in Figure 2, activin A remarkably promoted the phagocytosis and pinocytosis of RAW264.7 cells.

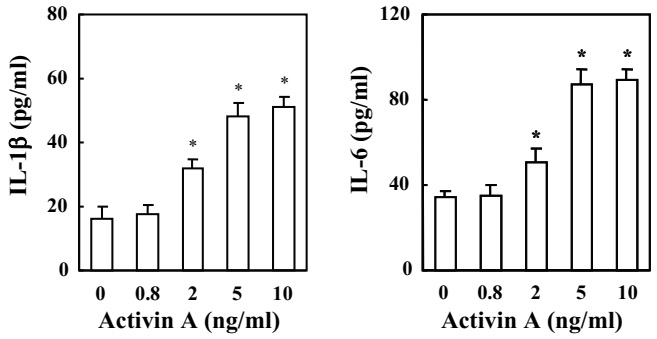

Figure 1. Assay of IL-1β and IL-6 levels from RAW264.7 cells. Mouse macrophage RAW264.7 cells were incubated with or without activin A (0.8-10 ng/ml). IL-1β and IL-6 levels in the supernatant of cultured macrophages were examined by ELISA. *$p < 0.01$, compared with control. All values are presented as mean ± SD of three independent experiments.

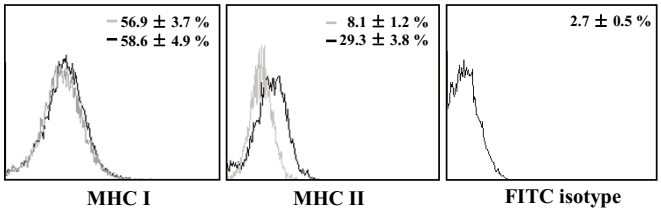

Figure 3. Flow cytometric analysis of MHC I and MHC II expressions on the surface of RAW264.7 cells. RAW264.7 cells were incubated overnight in the absence (—) or presence of 5 ng/ml activin A (▬). The cells were centrifuged, resuspended in 2% FCS/PBS, and incubated with 1 μg of FITC-conjugated anti-mouse MHC I or MHC II antibody for flow cytometric analysis. Values (mean ± SD of three independent experiments) in the profile showed the percent of positive fluorescence cells. A representative experiment of three performed is shown.

Activin A influences the expressions of MHC I and MHC II on RAW264.7 cells

MHC I and MHC II are the important surface molecules of macrophage, and involved in presentation of endogenous and exogenous antigens, respectively. To explore possible effect of activin A on presentation of antigens, the expressions of both MHC I and MHC II on the surface of RAW264.7 cells were examined by flow cytometry. The results showed that activin A obviously up-regulated MHC II expression on the surface of RAW264.7 cells, whereas MHC I expression was not altered (Figure 3).

Activin A up-regulates CD80 expression on RAW264.7 cells

To confirm the role of activin A in macrophage activation, we examined the expression of CD80, a classical surface marker of activated macrophages (14), on RAW264.7 cells. We found that CD80 expression was augmented on RAW264.7 macrophages when treated with activin A (Figure 4). These results suggested that activin A might promote the activation of RAW264.7 cells directly.

Activin A has no effect on the proliferation of RAW264.7 cells

In this study, we also examined the effect of activin A on the proliferation of RAW264.7 cells by MTT colorimetric method. The results revealed that there was no significant difference in proliferation of RAW264.7 cells treated with or without activin A. These findings suggested that activin A induced the activation of mouse RAW264.7 macrophages, but had no effect on macrophage proliferation.

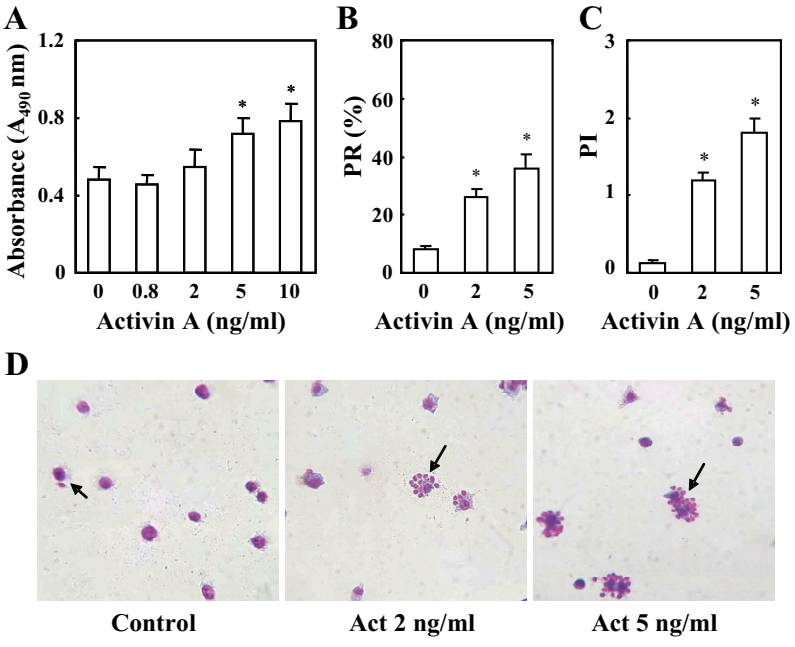

Figure 2. Analysis of pinocytosis and phagocytosis of RAW264.7 cells *in vitro*. Pinocytosis of RAW264.7 cells was evaluated by neutral red assay after untreated or treated with activin A *in vitro* (A). The phagocytosis ratio (PR) (B) and index (PI) (C) of RAW264.7 cells to cRBC were examined after untreated or treated with activin A *in vitro*. All values are presented as mean ± SD of three independent experiments. *$p < 0.01$, compared with control. (D) RAW264.7 cells phagocytosing cRBC were stained with Wright-Giemsa dye and observed with light microscope (×200). The arrows represent the phagocytosed cRBC.

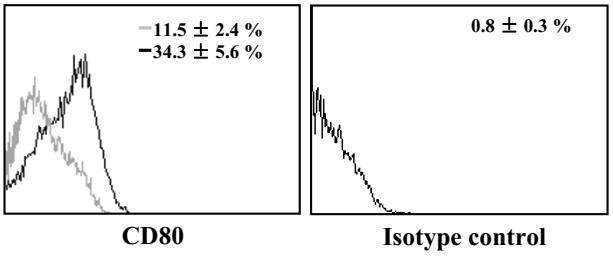

Figure 4. Detection of CD80 on the surface of RAW264.7 cells by flow cytometry. RAW264.7 cells were incubated overnight in the absence (—) or presence of 5 ng/ml activin A (▬). The expressions of CD80 was detected. Values (mean ± SD of three independent experiments) in the profile showed the percent of positive fluorescence cells. A representative experiment of three performed is shown.

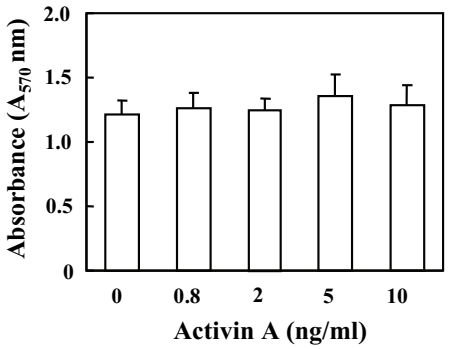

Figure 5. Proliferation assay of RAW264.7 cells. RAW264.7 cells in 96-well culture plates were incubated for 24 h in the absence or presence of activin A (0.8-10 ng/ml). The cells were stained with MTT for 4 h. Media were removed and the formazan crystala was dissolved by adding dimethylsulfoxide. Absorbance was detected at 570 nm to express the cell viabilities.

Discussion

Activin is a multifunctional member of TGF-β superfamily (15-17). Homo- or hetero-dimerization of two inhibin β subunits (βA and βB) form three types of activins, activin A (βAβA), activin B (βBβB) and activin AB (βAβB). Activin A plays a critical role in many physiological processes, including regulation of secretion of follicle-stimulating hormone (FSH) from anterior pituitary, differentiation of erythroblasts and embryonic development (18-20).

IL-1β, secreted from monocytes/macrophages, is a classical mediator of the host inflammatory response to infections and other inflammatory stimuli. IL-6 is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and stimulates the synthesis of acute-phase proteins and thus contributes to the systemic effects of inflammation. In this study, we found that activin A markedly increased IL-1β and IL-6 production from mouse RAW264.7 macrophages. Our previous study has reported that activin A can promote other inflammatory mediators, NO production from mouse peritoneal macrophages *in vitro*. These studies suggest that activin A may induce inflammatory mediator release from macrophages in the early stage of inflammation.

In addition to secreting inflammatory mediators, macrophages can also phagocytose large antigen particles, including various pathogenic microorganism, apoptotic cells and tumor cells. After phagocytosing the microbes, macrophages lyse and digest them inside the phagolysosome *via* oxygen-dependent or oxygen-independent mechanisms. Pinocytosis is also an important function of macrophage, by which tiny vesicle will be taken into the interior of the cytoplasm of macrophages. By phagocytosis and pinocytosis, macrophages play an important role in innate immune responses (21, 22). In the present study, we found that activin A promoted phagocytosis and pinocytosis of macrophages to cRBC and neutral red, respectively, suggesting that by up-regulating mouse macrophage activities, activin A may play critical roles in host inflammation response.

Macrophages are involved in inspiring the primary response of the adaptive immune system *via* processing and presenting antigens. Since activin A can regulate phagocytic and pinocytic activities of macrophages, it may also influence presentation of antigens. MHC II is mainly expressed on antigen-presenting cell surface, such as macrophages, B cells and DCs, and designed to stably bind and present fragments of exogenous antigen to T cells. MHC I is widely expressed on various cell surface and mediates presentation of endogenous antigen. The present study revealed that activin A up-regulated MHC II expression, but did not influence MHC I expression on the surface of mouse macrophage RAW264.7 cells, indicating that activin A might promote antigen-presenting ability of mouse macrophages to exogenous antigens.

Furthermore, our data revealed that expression of macrophage activated marker CD80 on the surface of RAW264.7 cells was greatly induced by activin A. The activated macrophages also highly express MHC II molecules (23). Our data showed that activin A also promoted the MHC II expression on the surface of mouse macrophage RAW264.7 cells. But there was no significant change in RAW264.7 cell proliferation after treated with activin A. These findings suggest that activin A induce the activation of mouse RAW264.7 macrophages without influencing cell proliferation.

Macrophages are present in all tissues of the human body where they normally assist in guarding against invading pathogens and regulate tissue remodeling. However, they are also known to accumulate in large numbers in inflammation sites, and might cause tissue injury. Our present data provide further insight that activin A plays direct roles in the regulation of primary macrophage activation in the early phase of inflammation.

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