Activin A Stimulates Type IV Collagenase (Matrix Metalloproteinase-2) Production in Mouse Peritoneal Macrophages

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Activin A Stimulates Type IV Collagenase (Matrix Metalloproteinase-2) Production in Mouse Peritoneal Macrophages

Kenji Ogawa, Masayuki Funaba, Lawrence S. Mathews, and Takeo Mizutani

The role of activin, a dimer of inhibin β subunit, in mouse peritoneal macrophages was evaluated. Activin activity in the cultured macrophages was augmented in response to activation by LPS. In Western blot analysis, immunoreactive activin A was detected in the culture medium only when the macrophages were stimulated by LPS. Although mRNA expression of βA subunit was detected, that of α and βB subunit was not found in macrophages by reverse RT-PCR. The activin βA mRNA level was increased in macrophages by LPS, suggesting that the activin production augmented by LPS is regulated at the mRNA level of the βA gene. The mRNAs of four activin receptors (ActRI, ActRIB, ActRII, and ActRIIB) were also detected in the peritoneal macrophages, and the mRNA levels, except for ActRIB, were decreased during the LPS treatment. Exogenous activin A stimulated the mRNA expression and gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) in macrophages in both the presence and the absence of LPS. In contrast, activin did not affect the production of MMP-9 in macrophages. These results suggested that 1) mouse peritoneal macrophages produced activin A; 2) expression of activin A was enhanced with activation of the macrophages; 3) the macrophages also expressed activin receptors; and 4) exogenous activin A stimulated MMP-2 expression and activity, implicating activin A as a positive regulator of MMP-2 expression. Considering that MMP-2 constitutes the rate-limiting proteinase governing the degradation of basement membrane collagens, activin A may be involved in migration and infiltration of macrophages through the basement membrane in an inflammatory state.

A ctivins, which are homo- or heterodimers of inhibin βA and βB subunits and members of the TGF-β superfamily, are local regulators of cell growth and differentiation. Diverse physiological functions of activins include, in the neural and endocrine tissues, stimulation of follicle-stimulating hormone (1, 2) and prolactin (3) secretion from the pituitary cells, inhibition of pituitary growth hormone secretion (4), nerve cell survival and differentiation (5, 6), neural differentiation in Xenopus embryo (7, 8), stimulation of insulin secretion from pancreatic islets (9), and stimulation of steroidogenesis in gonadal cells (10–12).

In addition to extensive knowledge about the role of activin in neural and endocrine tissues, the expression and function of activins in immune system have been reported, although the evidence for the possible role of activin as an autocrine/paracrine factor in circulating monocytes. However, the production of cytokines, including structurally related TGF-β1 in alveolar macrophages, was quite distinct from that in blood monocytes (23), suggesting differences in the expression and function of activin in tissue macrophages. Here, we showed that 1) expression of activin A in mouse peritoneal macrophages was increased with the activation by thiglycolate (TGC) injection in vivo or by LPS treatment in vitro; 2) gene transcripts of both type I and type II activin receptors were also expressed, and the type II activin receptors were down-regulated in response to the activation; and 3) activin A stimulated expression of matrix metalloproteinase-2 (MMP-2) in mouse peritoneal macrophages. Our results suggest that activin A acts as a positive regulator of MMP-2 production by peritoneal macrophages.

Materials and Methods

Reagents

RPMI 1640 medium supplemented with 2 mM glutamine was purchased from Iwaki Glass (Chiba, Japan). FBS was purchased from Otsuka International (Ontario, Canada). Serum-free S-Clone SF-02 medium was purchased from Sanko Junyaku (Tokyo, Japan). Recombinant human activin A and recombinant human follistatin were provided by the National Hormone and Pituitary Program (Rockville, MD). LPS from Escherichia coli 0111:B4 was purchased from Sigma (St. Louis, MO).

Animals

Specific patogen-free BALB/c mice were obtained from Japan SLC (Shizuoka, Japan) and maintained in the Laboratory Animal Research Center, RIKEN. Female mice were used at 8–12 wk of age.

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2 Address correspondence and reprint requests to Dr. Kenji Ogawa, Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.
Collection and cultivation of macrophages

Resident peritoneal macrophages were obtained by lavage of the peritoneal cavity of mice with 8 ml of cold sterile saline. TG-C elicited peritoneal macrophages were obtained using mice injected 4 days previously with 2 ml of a sterile 3% Brewer thioglycollate broth (Difco, Detroit, MI). The cells were collected by centrifugation, washed, and resuspended at 1.5 × 10^6 cells/ml in RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated (56°C, 30 min) FBS. Cells were seeded either in 24-well plates (Iwaki Glass) at 2 ml/well or six-well plates (Iwaki Glass) at 5 ml/well, and allowed to adhere to tissue culture plates for 2 h at 37°C before gentle rinsing to remove nonadherent cells. In the resultant macrophage monolayer >98% of the cells were MAC-1 positive (data not shown). Then the cultures were immediately treated with reagents in 2 ml/well (24-well plate) or 3 ml/well (six-well plate) of serum-free S-Clone SF-02 medium (Sanko Junyaku). For controls, macrophages were incubated in medium alone. LPS (0–100 ng/ml) or activin A (125 ng/ml) was added to the culture medium.

Bioassay for activin activity

Activin activity in macrophage-conditioned medium was assayed by erythropoietin differentiation assay using mouse erythroleukemia F5–5.fl cells. For Western blot analysis, TGC-treated macrophages were cultured for 72 h with or without 100 ng/ml of LPS. TCA-precipitated supernatant were directly used for Western blotting.

Bioassay for activin activity

Serially diluted samples were added to F5–5.fl cells at a cell density of 1000 cells/well and allowed to adhere to tissue culture plates for 2 h at 37°C before gentle rinsing to remove nonadherent cells. In the resultant macrophage monolayer >98% of the cells were MAC-1 positive (data not shown). Then the cultures were immediately treated with reagents in 1 ml/well (24-well plate) or 3 ml/well (six-well plate) of serum-free S-Clone SF-02 medium (Sanko Junyaku). For controls, macrophages were incubated in medium alone. LPS (0–100 ng/ml) or activin A (125 ng/ml) was added to the culture medium.

RNA isolation and cDNA synthesis

Total RNA from freshly prepared macrophage monolayers and mouse ovaries was isolated using the RNAsena Total RNA Isolation System (Promega, Madison, WI). Mouse ovary was excised from immature female animals (4 wk of age) 48 h after ip injection of 7.5 IU of pregnant mare serum gonadotropin (PMSG; Serotropin, Teikoku Zoki, Tokyo, Japan). One microgram of the recovered RNA was treated with RNase-free DNase I (Life Technologies, Gaithersburg, MD) to remove the residual DNA and reverse transcribed in a 21-μl volume reaction with oligo(dT) primer using the Superscript Preamplification System (Life Technologies) to generate first-strand cDNA. Products were diluted to a final volume of 400 μl. Diluted cDNA was stored at −80°C until used for PCR.

Polymerase chain reaction

The oligonucleotides used for PCR to detect the expression of activin subunit (25), follistatin (26), type I and type II receptors for activin (27–30), Mmp-2, Mmp-3, and G3PDH (33) are shown in Table I. PCR were performed in a total volume of 50 μl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 1 μM of each primer, 1.75 U of a DNA polymerase mixture of the Expand High-Fidelity PCR System (Roche, Indianapolis, IN), and 5 μl of previously diluted RT reaction. The thermal cycling parameters consisted of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 45 s for 35 cycles. The PCR products were separated on 1.5% agarose gels in 0.5 x TBE and visualized with ethidium bromide.

Competitive RT-PCR

Competitor templates were constructed for activin βA subunit, follistatin, four activin receptors, two MMPs, and G3PDH genes (Table II). A deletional mutation was synthesized by overlap extension PCR (βA subunit, ActRi, ActRii, ActRibi, Mmp-2, Mmp-3, G3PDH) or restriction endonuclease digestion (follistatin, ActRibi) of native PCR product and cloned into pBluescript vector. Each deletional mutant was then amplified by PCR with the original primers, and the resultant competitor template was purified by HPLC using TSKgel DNA-NPR column (4.6 × 75 mm; Tosoh, Tokyo, Japan), and the concentration was determined by absorbance at 260 nm. A constant amount of competitor template was amplified with reverse-transcribed samples or varying amounts of the target cDNA standard. Following amplification, the competitor and the target products were separated and analyzed quantitatively by HPLC using the protocol described above.

Table I. Sequences of oligonucleotides used for PCR

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<th>Transcript</th>
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<th>Product (bp)</th>
<th>Ref.</th>
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<td>33</td>
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<td></td>
<td>3’</td>
<td>982–1001</td>
<td>TAC TCC TTG GAG GGC ATG TA</td>
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Gelatin zymography

Macrophase-conditioned medium was subjected to gel electrophoresis (34) with some modifications. The samples were applied without reduction to 10% polyacrylamide gel impregnated with 0.25 mg/ml gelatin (Sigma). After electrophoresis, the gel was washed in washing buffer (50 mM Tris-HCl (pH 7.5), 5 mM CaCl2, 1 mM ZnCl2, and 2.5% Triton X-100) for 30 min at room temperature, and then incubated overnight at 37°C with shaking in the same buffer, except that 1% Triton X-100 was added. The gel was stained with a solution of 0.1% Coomassie Brilliant Blue R-250. In this assay, clear zones against the blue background indicate the presence of gelatinolytic activity. The gelatinolytic activity was quantified using densitometric analysis by NIH Image. For inhibition studies, gel slices were incubated overnight at 37°C in the presence of 20 mM EDTA.

Statistical analysis

Data were presented as the mean ± SD. Comparisons between groups were conducted by Student’s t test. For all analyses, p < 0.05 was considered significant.

Results

Expression of activin A is induced in mouse peritoneal macrophages in response to activation

To evaluate the role of activin in mouse peritoneal macrophages, activin activity in the culture medium of macrophages with or without LPS, a potent inflammatory stimulus, was measured by erythroid differentiation assay. The LPS treatment significantly increased activin activity in culture medium of resident macrophages (Fig. 1A, left). The LPS-induced activin activity was also detected in TGC-elicited macrophages, although the culture medium of TGC-elicited macrophages had higher activin activity than that of resident macrophages, even in the absence of LPS stimulation (Fig. 1A, right). Activin activity of the culture medium of TGC-elicited macrophages peaked at 72 h during LPS treatment (Fig. 1B), and LPS increased activin activity in a dose-dependent manner (Fig. 1C). The LPS-induced activin activity was neutralized by addition of follistatin, an activin-binding protein that neutralizes the action of activin A in many biological systems (35–38), or anti-activin A neutralizing mAb in erythroid differentiation assay (Fig. 1D). In Western blot analysis, immunoreactive activin A was detected in the culture supernatant as a 25-kDa band only when the peritoneal macrophages were treated with LPS (Fig. 1E). These results suggest that bioactive activin protein is produced in mouse peritoneal macrophages activated either by TGC injection in vivo or by LPS treatment in vitro.

Next, the expression of activin in peritoneal macrophages was examined at the mRNA level (Fig. 2). The mRNA expression of activin subunit and follistatin in macrophages was analyzed by RT-PCR. RNA from PMSG-primed mouse ovary, a known tissue expressing activin/inhibin and follistatin (1), was used as a positive control. RT-PCR of TGC-elicited peritoneal macrophage RNA yielded inhibin βA subunit and follistatin PCR products, but not products of inhibit α and βB subunits (Fig. 2A). Considering detection of activin activity and immunoreactive activin in the culture medium of the peritoneal macrophages, the RT-PCR results suggested that the peritoneal macrophages expressed activin A, a homodimer of inhibit βA subunit.

We also quantitatively measured the mRNA levels of activin and follistatin by competitive RT-PCR. A representative standard curve from competitive PCR for activin βA is shown in Fig. 2B; when a constant amount (5 × 10^-3 amol) of competitor was co-amplified with varying concentrations of activin βA cDNA, the intensity of the band corresponding to competitor was decreased with increasing activin βA cDNA (left), and a plot of the competitor to target ratio against the concentration of activin βA cDNA exhibited a linear relationship (right). Standard curves for follistatin and G3PDH cDNAs were also obtained (data not shown), and the mRNA level was expressed as the ratio to G3PDH. The activin βA mRNA level was evaluated by LPS treatment (Fig. 2C, left), suggesting that the increased production of activin A protein during LPS stimulation was regulated at the mRNA level. Although the LPS treatment also elevated follistatin mRNA level (Fig. 2C, right), the follistatin mRNA level was about 50 times lower than the activin βA mRNA level.

Type II activin receptor mRNAs are decreased with activation of peritoneal macrophages

In addition to the evaluation of activin expression in peritoneal macrophages, we further examined expression of receptors for activin. RT-PCR analysis revealed the PCR products of two type I receptors (ActRI and ActRIB) and two type II receptors (ActRII and ActRIIB) for activin at the expected sizes (Fig. 3A), suggesting that mouse peritoneal macrophages expressed not only activin A but also activin receptors. Quantitative RT-PCR revealed that the mRNA levels of ActRI, ActRII, and ActRIB were significantly decreased by LPS treatment, whereas the ActRIB mRNA level was not changed (Fig. 3B).

Table II. Competitor constructs by deleting cDNA segment used for competitive RT-PCR

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<td>372 bp</td>
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Activin A stimulates the expression of MMP-2 in peritoneal macrophages

We explored the role of activin in peritoneal macrophages. Activin A expression was increased with the activation of peritoneal macrophages, which would lead to migration and infiltration through degradation of extracellular matrix. Thus, the expression of type IV collagenases was checked: mRNA levels by competitive RT-PCR, and protein levels by gelatin zymography. The effect of exogenous activin A treatment of peritoneal macrophages on type IV collagenase expression was examined at 48 h of LPS treatment, because the mRNA level of MMP-9 tended to decrease after 48 h of LPS treatment, in contrast to an increase in the MMP-2 mRNA level up to 72 h (Fig. 4A).

Exogenous activin A elevated the MMP-2 mRNA level in peritoneal macrophages, although the extent of the increase was smaller in the presence of LPS (63% increase) than in the absence of LPS (405% increase; Fig. 4B). In contrast, activin A did not affect MMP-9 mRNA levels in peritoneal macrophages regardless of LPS treatment (Fig. 4B). Consistent with the results at the mRNA level, gelatinase activity of MMP-2 was increased in peritoneal macrophages by exogenous activin A, and the extent was smaller in the presence of LPS (Fig. 4C). MMP-9 activity was not changed by the addition of activin A (Fig. 4C).
Discussion

We have shown that mouse peritoneal macrophages increased the production of activin A with their activation; in contrast, type II activin receptors, activin receptors responsible for activin binding (39), were decreased. Furthermore, exogenous activin A enhanced the expression and gelatinase activity of MMP-2 in peritoneal macrophages. Our results suggest that activin A as a regulator is involved in migration and infiltration of the activated macrophages through the stimulation of MMP-2 expression.

RT-PCR revealed inhibin βA subunit mRNA, but not inhibin α and βB subunits, in peritoneal macrophages. In Western blot analysis, immunoreactive activin in the macrophage-conditioned medium was found at the same molecular size as recombinant activin A. Furthermore, the erythroid differentiation assay, a known assay for evaluating activin activity (24), showed activin activity in the macrophage-conditioned medium that was neutralized by addition of follistatin or anti-activin A-neutralizing mAb. These results indicated that mouse peritoneal macrophages produced activin A, but not inhibin, consistent with previous results obtained from the same lineage of cells. Peripheral blood monocytes produced activin A, which was shown by detection of activin βA mRNA and immunoreactive activin A molecules and by erythroid differentiation assay of the culture medium (18–20). In addition, alveolar macrophages expressed activin A, which was revealed by immunohistochemistry (40). Furthermore, activin A was purified from culture medium of a murine macrophage cell line, P388D1 (41).

Treatment with LPS or GM-CSF increased activin production in monocytes (18–20). Consistent with the previous results, activin A production increased in a dose-dependent manner in the presence of LPS or GM-CSF (18–20). Furthermore, the presence of activin A increased the expression of activin receptors in peritoneal macrophages (39). These results suggest that activin A as a regulator is involved in migration and infiltration of the activated macrophages through the stimulation of MMP-2 expression.

FIGURE 3. Changes in activin receptor mRNA levels in peritoneal exudate macrophages in response to stimulation by LPS. A, Expression of type I and type II receptors for activin in mouse peritoneal exudate macrophages. The mRNA expression of receptors for activin in TGC-elicited mouse peritoneal macrophages was compared with that in PMSG-primed immature mouse ovary as a positive control. Lane 1, Macrophages; lane 2, ovary; lane 3, no RT control of macrophage. B, Effect of LPS on the expression of activin receptors mRNA in mouse peritoneal macrophages. Peritoneal exudate macrophages were incubated for 72 h with 100 ng/ml of LPS, when total RNA was isolated and subjected to competitive RT-PCR. The mRNA level was expressed as a ratio to G3PDH mRNA (n = 3).

FIGURE 4. Effect of activin on type IV collagenase expression in macrophages. A, Time-course changes in LPS-stimulated MMP-2 and MMP-9 mRNA expression in macrophages. The cDNA samples from the macrophages treated with 100 ng/ml of LPS for 72 h were subjected to competitive RT-PCR. The mRNA was expressed as a ratio to G3PDH mRNA (n = 3). B, Effect of activin on the expression of MMP-2 and MMP-9 mRNA in mouse peritoneal macrophages. The mRNA was expressed as a ratio to G3PDH mRNA (n = 3). C, Effect of activin on the production of type IV collagenase by mouse peritoneal macrophage. The mRNA was expressed as a ratio to G3PDH mRNA (n = 3). The figure shows a representative experiment of three independent experiments.
expression was increased by LPS treatment in mouse peritoneal macrophages. Although the LPS treatment also elevated the follistatin mRNA level, the extent was less remarkable. Considering that net activin activity evaluated by erythroid differentiation assay was increased, the increased follistatin expression would not imply the neutralization of activin, but may result from increased activin expression. Previous results showed that activin was responsible for increased follistatin expression in rat anterior pituitary after ovariectomy (42).

These changes in expression and activity of activin with the LPS stimulation were different from those in structurally related TGF-β RNA transcripts. The TGF-β mRNA level was not changed by LPS stimulation of human monocytes (43). Zhou et al. (44) also showed that the TGF-β1 mRNA level was rapidly decreased within 3 h after LPS treatment of PBMC. Furthermore, we observed that the LPS treatment suppressed TGF-β1 mRNA to 20% or less of the control value (our unpublished observations). The distinct expression in response to LPS stimulation suggested the different function of activin from that of TGF-β in peritoneal macrophages.

This study revealed that mouse peritoneal macrophages expressed not only activin A but also the receptors, suggesting that the macrophages could also be targets of activins. Activin was responsible for the induction and activation of MMP-2 in peritoneal macrophages; exogenous activin A increased both the mRNA level and the net gelatinase activity of MMP-2 without affecting MMP-9 expression. Similar evidence has been detected in culture medium of villous explants from human placentas; activin A specifically induced expression of MMP-2 (45). Consistent with the previous results (46), LPS treatment stimulated both MMP-2 and MMP-9 production, and exogenous activin A further increased MMP-2 expression in LPS-stimulated peritoneal macrophages, although the extent of the increase was less than that in the macrophages without LPS stimulation. The lesser effect on the MMP-2 production may result from the decrease in expression of type II activin receptors for activin A in human monocytes (46). Considering that MMP-2 constitutes the secretion of follicle stimulating hormone.

Acknowledgments
We thank the National Hormone and Pituitary Program for providing the human recombinant activin A and human recombinant follistatin. We are grateful to Satoshi Onawa and Masaki Kumai from Brain Science and Life Technology Research Foundation, and to Tsutomu Oowada, Kyouchi Uchiumi, and Akira Ozaki from Laboratory Animal Research Center, RIKEN, for their expert care of the laboratory animals.

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