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Atrial Natriuretic Peptide Polarizes Human Dendritic Cells Toward a Th2-Promoting Phenotype Through Its Receptor Guanylyl Cyclase-Coupled Receptor A

Rimpei Morita,* Naoya Ukyo,* Mayumi Furuya,† Takashi Uchiyama,* and Toshiyuki Hori2*

Atrial natriuretic peptide (ANP) is a cardiovascular hormone secreted mainly by the cardiac atria and regulates the volume-pressure homeostasis. The action of ANP is mediated by its receptor, guanylyl cyclase-coupled receptor A (GC-A). In this study, we explored the possibility that ANP and GC-A may play a role in the dendritic cell (DC)-mediated immune regulation. We first examined the expression of GC-A in human monocyte-derived DCs in comparison with monocytes and found that DCs but not monocytes express GC-A at both the mRNA and protein levels. DCs responded to ANP with an increase in intracellular cGMP in a dose-dependent manner, indicating that GC-A expressed on DCs is functional. Furthermore, treatment of DCs with ANP decreased production of IL-12 and TNF-α and conversely increased that of IL-10 upon stimulation with LPS. In accordance with this change of cytokine production, DCs treated with ANP plus LPS promoted differentiation of naïve CD4+ T cells into a Th2 phenotype. Finally, we presented evidence that ANP affected cytokine production of fresh whole blood stimulated with LPS in line with the above-mentioned results. These results indicate that ANP polarizes human DCs toward a Th2-promoting phenotype through GC-A and thus can regulate immune responses. The Journal of Immunology, 2003, 170: 5869–5875.
balance of cytokine production is deeply influenced by the mode of DC maturation, the actions of ANP on DCs need to be defined to better understand the pathophysiology of CHF and the elusive link between the immune system and body fluid regulation.

In the present study, we examined the possible involvement of ANP and its receptor GC-A in DC-mediated immune regulation. We show that human DCs but not monocytes expressed the functional ANP receptor GC-A and responded to ANP with an increase in intracellular cGMP in a dose-dependent manner. We also present data indicating that ANP treatment changes the cytokine production pattern of DCs stimulated with LPS, and that these DCs polarize naive CD4\(^+\) T cells toward a Th2 phenotype.

**Materials and Methods**

**Preparation of human DCs and their culture**

Peripheral blooduffy coats were obtained from healthy human donors (kindly provided by Kyoto Prefectural Red Cross Blood Center, Kyoto, Japan). PBMCs were isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, NJ), anti-CD14 (Miltenyi Biotec, Bergisch Gladbach, Germany) or by a 1 h adherence to 75 cm\(^2\) cell culture flasks (Iwaki, Tokyo, Japan) at 37°C. Immature DCs were generated by culturing monocytes at 2 \times 10^6 cells/ml for 7 days in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated FBS (Life Technologies, Paisley, U.K.), 10 mM HEPES (Sigma-Aldrich), 50 ng/ml human rGM-CSF (kindly provided by Novartis, Basel, Switzerland), and 20 ng/ml human rIL-4 (PeproTech, Rocky Hill, NJ). For activation and maturation, immature DCs were cultured with 1 \times 10^6 LPS (Escherichia coli) and 20 ng/ml human rIL-4 (PeproTech, Rocky Hill, NJ). After immunoanalysis: anti-HLA-DR (BD Biosciences, San Jose, CA), anti-CD40 (BD PharMingen, San Diego, CA), anti-CD80, anti-CD83, anti-CD86, and anti-GC-A expression, cells were stained with A-397, anti-human GC-A mAb (BD Biosciences), and analyzed with a FACScan (BD Biosciences). Stained cells were analyzed with a FACScan (BD Biosciences) using CellQuest software (BD Biosciences).

**Flow cytometric analysis of cell surface Ags**

The following FITC- or PE-conjugated mAbs were used for flow cytometric analysis: anti-HLA-DR (BD Biosciences, San Jose, CA), anti-CD40 (BD PharMingen, San Diego, CA), anti-CD80, anti-CD83, and anti-CD86, or an isotype-matched control Ig followed by incubation with FITC- or PE-conjugated mAbs: anti-HLA-DR, anti-CD40, anti-CD80, anti-CD83, anti-CD86, anti-GC-A, anti-CD80, and anti-GC-A mAb (BD Biosciences). After incubation with myeloperoxidase, permeabilized with PBS containing 2% FBS and 0.5% saponin (Sigma-Aldrich), 50 ng/ml human rGM-CSF (kindly provided by Novartis, Basel, Switzerland), and 20 ng/ml human rIL-4 (PeproTech, Rocky Hill, NJ). For activation and maturation, immature DCs were cultured with 1 \times 10^6 LPS (Escherichia coli, 0111:B4; Sigma-Aldrich) in the absence or presence of 10^–5 to 10^–6 M ANP (Peptide Institute, Osaka, Japan) or 10^–6 M CNP (Peptide Institute) for up to 48 h.

**RT-PCR for natriuretic peptide receptors and cytokines**

Total RNA was isolated from DCs with an RNA isolation kit, RNeasy (Qiagen, Hilden, Germany), and single-strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase using 1 \mu\text{g} total RNA and oligo(dT) primer. The primer pairs indicated in Table I were used (28–32).

PCR was performed in 35 cycles for natriuretic peptide receptors and cytokines and in 25 cycles for \beta\text{-}actin. PCR products were separated through 1.5 or 2% agarose gel, stained with ethidium bromide, and visualized with an UV transilluminator.

**Measurement of intracellular cGMP**

Cells (1 \times 10^6 cells/sample) were washed with prewarmed PBS three times and incubated in 50 \mu\text{M} RPMI 1640 with 0.5 mM 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor (Wako Pure Chemical, Tokyo, Japan), 10 mM HEPES, and 1 \mu\text{M} phosphoramidon (Nacalai Tesque, Kyoto, Japan) for 10 min at 37°C (33, 34). ANP or CNP was added to the final concentrations of 10^{-12} to 10^{-6} M and incubated for an additional 15 min at 37°C (33, 34). Cells were washed with prewarmed PBS, lysed, and subjected to the measurement of intracellular cGMP using a cGMP ELISA system (Amersham Pharmacia Biotech) according to the manufacturer’s instruction.

**Proliferation of allogeneic naive CD4\(^+\) T cells in response to activated DCs**

Naïve CD4\(^+\) T cells were purified from umbilical cord blood mononuclear cells of healthy neonates by MACS (Miltenyi Biotec) using a MACS CD4\(^+\) T cell isolation kit. CD45RA\(^+\) CD4\(^+\) cells accounted for >95% of the isolated cells. Monocyte-derived DCs that had been pretreated with the indicated reagents were washed thoroughly, irradiated (30 Gy), and cocultured with allogeneic naïve CD4\(^+\) T cells (1 \times 10^5 cells/well) in 96-well round bottom plates in quintuplicate for 6 days. Cells were pulsed with 0.5 \mu\text{Ci/well} [3H]thymidine (Amersham Pharmacia Biotech) for the last 8 h and harvested onto glass fiber filters. Incorporated radioactivity was counted by a liquid scintillation counter (Packard Instrument, Downers Grove, IL).

**Analysis of intracellular cytokine production**

Naïve CD4\(^+\) T cells (1 \times 10^5 cells/well) isolated from cord blood were cocultured in 24-well plates for 6 days with DCs (1 \times 10^5 cells/well) that had been pretreated with the indicated reagents and irradiated. After expansion in the presence of IL-2 (50 U/ml) for another 8 days, cells were collected and stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Calbiochem, La Jolla, CA) for 4 h (23). Brefeldin A (10 \mu\text{g/ml}) (Sigma-Aldrich) was added for the last 2 h. Cells were fixed with 2% formalin, permeabilized with PBS containing 2% FBS and 0.5% saponin, and then stained with FITC-anti-IFN-\gamma mAb and PE-anti-IL-4 mAb (BD Biosciences). Stained cells were analyzed with a FACScan (BD Biosciences).

**Cytokine production of fresh whole blood stimulated with LPS**

Cytokine production from fresh human whole blood was determined according to the method previously described (11). Briefly, heparinized venous blood from healthy volunteers was diluted 5-fold with RPMI 1640 supplemented with penicillin-streptomycin and heparin (2 IU/ml final concentration) and incubated in the presence of LPS alone or ANP plus LPS for 24 h. Then, cells were sedimented, and the culture supernatants were assayed for cytokine production by ELISA.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer Sequence</th>
<th>PCR Product (bp)</th>
<th>Annealing Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-A</td>
<td>Sense 5’–GGGAACCTCAGTTACGATCCAAC–3’</td>
<td>1163</td>
<td>55</td>
<td>28</td>
</tr>
<tr>
<td>GC-B</td>
<td>Sense 5’–ATGAAGGCCCAAGGACACTG–3’</td>
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<td>49</td>
<td>Original</td>
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<tr>
<td>GC-B</td>
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<td></td>
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<tr>
<td>NRP-C</td>
<td>Sense 5’–GAAGGATTATGCGCGCGGAGTGCCTC–3’</td>
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<td>66</td>
<td>29</td>
</tr>
<tr>
<td>IL-10</td>
<td>Sense 5’–ATGCCCGCCAGTGGACGAGGAAAGC–3’</td>
<td>352</td>
<td>61</td>
<td>30</td>
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<tr>
<td>IL-12 p35</td>
<td>Sense 5’–AAGGCCGCTGGTCAGCTATTCATCCA–3’</td>
<td>352</td>
<td>61</td>
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<tr>
<td>IL-12 p40</td>
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<td>54</td>
<td>31</td>
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<tr>
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<td>58</td>
<td>30</td>
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<tr>
<td>β-Actin</td>
<td>Sense 5’–ATCTATCGTTAAGGAGGTTCTT–3’</td>
<td>314</td>
<td>60</td>
<td>32</td>
</tr>
</tbody>
</table>

Table I. Oligonucleotide primers for natriuretic peptide receptors and cytokines
Quantification of cytokines by ELISA
Cytokine concentrations in the supernatants were measured by the sandwich ELISA using matched-paired Abs specific for IL-4, IL-12 p70 (BD PharMingen), IL-10, IFN-γ, and TNF-α (BioSource International), respectively, according to the manufacturer’s instructions.

Statistical analysis
Statistical analyses were performed by Student’s t test or paired t test. Values of p < 0.05 were considered to be statistically significant.

Results
Immature DCs but not monocytes express GC-A
Natriuretic peptide receptors consist of GC-A, GC-B, and natriuretic peptide receptor C (NPR-C) that is a clearance receptor lacking substantial cytoplasmic domain (1, 3). To examine whether human DCs express any of these natriuretic peptide receptors, we first conducted RT-PCR analysis with monocytes and monocyte-derived immature DCs. As shown in Fig. 1A, mRNA expression of none of the natriuretic peptide receptors was detected in monocytes as had been previously reported (13). In contrast, monocyte-derived immature DCs clearly expressed mRNA of GC-A but not GC-B or NPR-C. Human placenta cDNA was used for a positive control for natriuretic peptide receptor mRNA (35, 36). Flow cytometric analysis using anti-human GC-A mAb, A-397, also revealed that expression of GC-A was induced after differentiation of monocytes into DCs (Fig. 1B). These results demonstrate that human DCs express GC-A at both the mRNA and protein levels.

ANP receptor GC-A expressed on DCs is functional
To determine whether GC-A expressed on DCs was functional, we next measured intracellular levels of cGMP in immature DCs as well as in monocytes after exposure to ANP or CNP. We chose CNP as a negative control because it is known that ANP binds to GC-A but not to GC-B while CNP binds to GC-B but not to GC-A (1, 2, 35). Cells were treated with serial dilutions of ANP or CNP (10^{-12}–10^{-6} M) for 15 min in the presence of 3-isobutyl-1-methylxanthine (0.5 mM) and then intracellular levels of cGMP were measured by ELISA. As shown in Fig. 2, intracellular levels of cGMP in immature DCs were increased after treatment with ANP in a dose-dependent manner while CNP had no effects, indicating that GC-A expressed on DCs is functional. This biological response was detected even at low concentrations such as at 10^{-12} M, which is well relevant for in vivo situations (37). As expected, neither ANP nor CNP induced an increase in cGMP in monocytes, which is consistent with the results that fresh monocytes express no natriuretic peptide receptors.

ANP elicits phenotypic changes of immature DCs
Obviously, the most important question was whether ANP has some immunological effects on DCs. Before going into the cytokine production and T cell stimulation, we examined the effects of ANP on the cell surface phenotype of DCs. Immature DCs were cultured with ANP and/or other reagents for 48 h and then subjected to the flow cytometric analysis. Treatment with ANP induced slight up-regulations of CD40, CD80, CD86, HLA-class I, HLA-DR, and CD83, whereas CNP (10^{-6} M) had no effects on these markers (Fig. 3A and data not shown). Conversely, stimulation with LPS resulted in marked up-regulations of these costimulatory molecules and HLAs. When added together with LPS, ANP had few effects on the phenotype of DCs (Fig. 3B).

ANP attenuates the capacity of LPS-stimulated DCs to induce proliferative response of allogeneic naive CD4^+ T cells
One of the major immunological functions of DCs is to prime naive T cells initiating Ag-specific immune response (21). We...
therefore tested whether ANP may regulate this DC function by comparing proliferative responses of allogeneic naive CD4+ T cells to ANP-treated and nontreated DCs in the absence or presence of LPS. Without LPS stimulation, ANP-treated DCs had relatively weak capacity to evoke proliferation of naive CD4+ T cells, which was comparable to that of nontreated immature DCs. In contrast, LPS-stimulated DCs induced a much higher proliferative response, and in this case, an addition of ANP showed marked inhibitory effects. This inhibition was evident especially in coculture longer than 4 days (Fig. 4A). As shown in Fig. 4B, DCs pretreated with ANP plus LPS induced only low levels of proliferation similar to nontreated immature DCs at 6 days, suggesting that ANP might interfere with the LPS-induced activating signals and alter the function of DCs. In these experiments, the incorporation of [methyl-3H]thymidine by naive CD4+ T cells alone or each DC preparation alone was <600 cpm and these values were subtracted from the raw data of MLRs.

**ANP changes the cytokine production of DCs in the presence of LPS**

DC-derived cytokines are known to play pivotal roles in the DC-T cell interaction (21–23). Accordingly, we examined whether ANP could change the cytokine production of DCs by RT-PCR for IL-10, IL-12 p35, IL-12 p40, and TNF-α. As shown in Fig. 5A, ANP alone induced expression in none of IL-10, IL-12 p35, IL-12 p40, or TNF-α mRNA in immature DCs. In LPS-stimulated DCs, however, ANP significantly reduced expression of IL-12 p35, IL-12 p40, and TNF-α mRNA and conversely augmented expression of IL-10 mRNA. To confirm these results, we performed ELISA for IL-12 p70, TNF-α, and IL-10 in the supernatants of LPS-stimulated DCs (24 h). As shown in Fig. 5B, an addition of ANP decreased the secretion of both IL-12 p70 and TNF-α while it increased that of IL-10 in dose-dependent manners. CNP included as a negative control had no effects on any cytokine production even at a concentration of 10−6 M.

**ANP polarizes immature DCs toward a Th2-promoting phenotype**

Because ANP altered the balance of IL-12 and IL-10 production by DCs, we next investigated whether ANP could polarize immature DCs toward a Th1- or Th2-promoting phenotype. To address this question, we cocultured allogeneic naive CD4+ T cells with DCs stimulated with LPS alone or ANP plus LPS. After interaction with DCs and following expansion in IL-2-containing medium, CD4+ T cells were analyzed for intracellular production of IFN-γ as well as IL-4 by flow cytometry. The results of two representative experiments are shown in Fig. 6A. We conducted five independent experiments and found that ANP treatment of DCs significantly decreased the percentage of IFN-γ-producing T cells and increased that of IL-4-producing T cells (Fig. 6B).

**ANP affects the cytokine production by fresh whole blood**

Finally, we examined the effect of ANP on cytokine production in simple ex vivo culture of fresh whole blood to confirm the physiological relevance of our results with monocyte-derived DCs. As shown in Fig. 7, an addition of ANP decreased the production of IFN-γ and conversely increased that of IL-4 and IL-10 by fresh
whole blood stimulated with LPS, which was consistent with the effect of ANP on DCs.

Discussion
In the present study, we report two novel findings. First, human DCs express functional ANP receptor, GC-A, and respond to ANP with an increase in intracellular cGMP. Secondly, ANP affects the cytokine production of DCs in the presence of LPS, and consequently polarizes DCs toward a Th2-promoting phenotype. During the past several years, evidence has been accumulating that ANP, a cardiovascular hormone, has certain immunoregulatory effects. For example, ANP has been reported to stimulate migration of human neutrophils (7), inhibit proliferation of rat thymocytes (8), enhance human NK cell cytotoxicity (9), and inhibit NO and TNF-α production of murine macrophages (10, 11). As to human monocytes, Sprenger et al. (13) previously reported that they neither expressed ANP receptors nor responded to ANP. However, DCs have never been investigated with regard to ANP and its receptor. As far as we know, this is the first report demonstrating that DCs express functional ANP receptor GC-A.

Because ANP and CNP specifically bind to GC-A and GC-B, respectively, and all three natriuretic peptides bind to NPR-C with virtually equal affinity (1, 2, 35), we used CNP as a control peptide. The data from RT-PCR for natriuretic peptide receptors clearly showed that immature DCs express GC-A alone among the three natriuretic peptide receptors. Furthermore, ANP but not CNP induced an increase in intracellular cGMP in DCs at such a low concentration as $10^{-12}$ M. Although we did not test it, it is possible that BNP, another natriuretic peptide, has some effects on DCs at high concentrations because BNP binds to GC-A with ~10-fold lower affinity than does ANP (35). However, it is most likely that among the three natriuretic peptides ANP mainly exerts actions on DCs via GC-A under physiological conditions.

Treatment of immature DCs with ANP alone resulted in slight up-regulations of costimulatory molecules as well as HLAs, but failed to induce cytokine production. Conversely in the presence of LPS, an addition of ANP attenuated the capacity of DCs to evoke the proliferative response of allogeneic naive CD4 T cells. Because ANP hardly affected the expression of the cell surface molecules in the presence of LPS, this decrease in allogeneic T cell stimulatory activity is likely to be due to changes of cytokine production of DCs. In fact, we showed that ANP treatment resulted in down-regulation of TNF-α that has a crucial role in T cell alloreactivity (38, 39) and up-regulation of IL-10 that suppresses IL-2 production and T cell proliferation (40).

It is now recognized that DCs can be instructed by various stimuli and mediators to differentiate into a polarized functional phenotype that promotes either Th1 or Th2 immune responses (21). LPS is known to interact with Toll-like receptor 4 on DCs and induce maturation into a Th1-promoting phenotype with high levels of IL-12 production (21, 41). Our data indicated that ANP could modulate the direction of DC maturation upon LPS stimulation. IL-12 is known as the most important cytokine for induction of a Th1 response, whereas IL-10 and several hormones, such as glucocorticoids and vitamin D₃, block IL-12 production to impair the ability of DCs to generate Th1 cells (20, 21, 24–26, 42). It is
unclear, however, whether the decrease in IL-12 production by LPS-stimulated DCs was brought by a direct action of ANP or mediated by the increase of IL-10 production. In any case, as expected from these findings, DCs treated with ANP plus LPS generated fewer IFN-γ-positive T cells and more IL-4-positive T cells from naive CD4+ T cells than did DCs treated with LPS alone. These results suggest that ANP polarizes immature DCs toward a Th2-promoting phenotype.

A number of studies have revealed the coincidence of cardiovascular disorders and immune responses. CHF and some types of cardiomyopathy with high levels of ANP have recently been recognized as syndromes involving not only cardiovascular dysfunction but also inflammatory process (14–16). It has been suggested that proinflammatory cytokines such as TNF-α, IL-1, and IL-6 play important roles in development of these disorders (14, 16). In particular, TNF-α presumably produced by activated macrophages and vascular endothelial cells has been shown to aggravate the hemodynamics of CHF (14, 16). As has been mentioned, clinical studies have demonstrated that targeted anti-TNF-α therapy with recombinant TNF-α receptor improves the functional status of patients with CHF (18). In this context, the role of ANP in cytokine regulation should be taken into consideration. ANP has been reported to suppress production of TNF-α in murine macrophages as previously mentioned (11). Moreover, we showed that ANP markedly inhibited TNF-α production and increased IL-10 production by DCs, which might have some contribution to restoration of the balance of cytokine production and to the control of inflammatory responses in cardiovascular disorders. Consistent with this result, high plasma levels of Th2 cytokines such as IL-4, IL-6, and IL-10 have been observed in patients with CHF (19, 43), which may be relevant to our data that ANP itself polarizes DCs toward a Th2-promoting phenotype.

We showed that ANP affects the cytokine production by fresh whole blood in line with the effect of ANP on monocyte-derived DCs. Furthermore, preliminary experiments have indicated that GC-A is also expressed on human blood DCs after a short-term (12 h) culture (R. Morita, unpublished data). Taken together, it is strongly suggested that the observation described in this study has certain physiological relevance. Considering that GC-A is expressed on macrophages (2) and DCs as we report but not on monocytes, ANP is likely to exert its immunoregulatory actions in tissue microenvironments, where the paracrine or autocrine mechanism of ANP may take place (2, 10). Further studies are required to define the roles of ANP and GC-A in immune regulation in vivo and to better understand the cross-talk between the reno-cardiovascular system and the immune system.

Acknowledgments
We thank Dr. K. Inaba (Faculty of Science, Kyoto University, Kyoto, Japan) for her critical reading of the manuscript and Dr. H. Hatayama (Adachi Hospital, Kyoto, Japan) for cord blood.

FIGURE 6. Flow cytometric analysis of intracellular IFN-γ and IL-4 in allogeneic naive CD4+ T cells after coculture with DCs pretreated with LPS alone or ANP plus LPS. Immature DCs were stimulated with LPS (1 μg/ml) in the presence or absence of ANP (10⁻¹⁰ M) for 24 h, washed, irradiated (30 Gy), and then cocultured with allogeneic naive CD4+ T cells. After 6 days, cells were split, expanded in the presence of IL-2 (50 U/ml) for another 8 days, and then analyzed for intracellular production of IFN-γ and IL-4 with a FACScan. A, The data of two representative experiments using samples from different donors are shown. B, The assay was repeated five times, and the percentage of IL-4- or IFN-γ-producing cells is shown for each sample. The differences in both IL-4- and IFN-γ-producing cells were statistically analyzed by paired t test. **, p < 0.01; *, p < 0.05.

FIGURE 7. Effects of ANP on cytokine production by fresh whole blood. Human whole blood from healthy donors was cultured for 24 h in medium alone or in medium containing LPS (1 μg/ml) or LPS plus ANP (10⁻¹⁰–10⁻⁶ M). The concentrations of IFN-γ, IL-4, and IL-10 in the culture supernatants were measured by ELISA. The results are shown as the mean and error bars represent ± SD of three experiments. ***, p < 0.01; and *, p < 0.05, compared with LPS-stimulated blood.
References