Adrenomedullin production is correlated with differentiation in human leukemia cell lines and peripheral blood monocytes

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Abstract We demonstrated that adrenomedullin (AM) is produced and secreted from human leukemia cell lines (THP-1 and HL-60) as well as peripheral blood granulocytes, lymphocytes, monocytes and monocyte-derived macrophages. Immunoreactive AM accumulated in the culture media of THP-1 and HL-60 cells increased according to their differentiation into macrophage-like cells. Retinoic acid exerted synergistic effects on AM secretion from THP-1 and HL-60 cells when administered with tumor necrosis factor-α, lipopolysaccharide or 12-*O*-tetradecanoyl phorbol-13-acetate. AM was shown to increase the scavenger receptor activity on THP-1 cells. Thus, monocytes/macrophages should be recognized as sources of AM, and the secreted AM may modulate the function of macrophages.

Key words: Adrenomedullin; Monocyte; Macrophage; Granulocyte; Differentiation

1. Introduction

Adrenomedullin (AM) is a potent vasorelaxant peptide originally isolated from extracts of human pheochromocytoma [1]. We have shown that cultured endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) actively produce and secrete AM into culture medium, and its production is augmented by interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) [2–5]. In an in vivo study, intravenous administration of LPS into rats elevated plasma AM concentration 20-fold and augmented AM gene expression in almost all tissues including blood vessels, lung and intestine [6]. The plasma AM levels in patients with septic shock also increased markedly compared with those in healthy volunteers [7]. These data suggest the possibility that AM contributes to the induction of refractory hypotension in septic shock.

On the other hand, monocytes/macrophages are activated by exposure to foreign bodies such as LPS, and then start to synthesize and secrete various cytokines, including IL-1 and TNF-α. These data suggest that monocytes/macrophages are another candidate for AM secreting cells in sepsis. To assess this possibility, the human monocytic cell line THP-1 was studied, since it is a highly differentiated monocytic cell line and expresses macrophage-like characteristics after stimulation with phorbol esters [8,9]. In addition, we examined a human myeloid leukemic cell line, HL-60, which can differentiate into macrophages and granulocytes by stimulation with phorbol ester and retinoic acid (RA), respectively [10,11]. In this study, we report that AM is produced and secreted from these cultured human leukemia cell lines after their differentiation as well as from peripheral blood monocytes and monocyte-derived macrophages.

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Abbreviations: AM, adrenomedullin; EC, endothelial cell; VSMC, vascular smooth muscle cell; IL-1, interleukin-1; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; RA, retinoic acid; IFN-γ, interferon-γ; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; FCS, fetal calf serum; RIA, radioimmunoassay; IR, immunoreactive; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; DiI-acLDL, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchorate acetylated low density lipoprotein

2. Materials and methods

2.1. Materials

Human AM (40–52) and its *N*-Tyr derivative were synthesized on a peptide synthesizer 431A (Applied Biosystems). The following materials were used: human interferon-γ (IFN-γ) (Pepro Tec Inc.), human recombinant IL-1β and TNF-α (R&D Systems), dexamethasone, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Wako Pure Chemicals), *Escherichia coli* LPS (serotype O26:B6) (Parsel+Lorei) and all-*trans* RA (Sigma). Acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindo-carbocyanine perchorate (DiI-acLDL) was purchased from Biomedical Technologies. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nikken Bio-Medical Laboratory. TPA and dexamethasone were first dissolved in ethanol, RA dissolved in dimethylsulfoxide, and then diluted with an incubation medium, DMEM containing 0.1% bovine serum albumin (BSA). LPS was dissolved in 0.9% NaCl solution and diluted with the incubation medium. The other substances were dissolved according to the producers' manuals and diluted with the incubation medium.

2.2. Cell culture

THP-1 and HL-60 cells were obtained from American Type Culture Collection and cultured in RPMI 1640 medium (Gibco) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Preparation of conditioned medium

THP-1 and HL-60 cells, grown for 4–5 days in 10 cm dishes (approximately 1×10^7 cells/dish), were washed twice with DMEM, and then 10 ml of DMEM containing 0.1% BSA and stimulants were added and incubated for 40 h at 37°C. To measure AM secretion from fully differentiated THP-1 cells, the cells were first incubated with TPA (10^{-8} M) for 3 days. Then, the differentiated cells were incubated for another 40 h after replacing the media. After incubation, acetic acid and Triton X-100 were added to the culture media (final concentration: 0.5 M and 0.002%), and the resulting solutions were heated at 100°C for 10 min to inactivate proteases. The incubates were extracted with pre-washed Sep-Pak C_{18} cartridges (Millipore Corp., Waters Division) as reported previously [6], since the levels of immunoreactive (IR) AM in the medium were too low to measure directly. The absorbed material was eluted with 60% acetonitrile in 0.1% trifluoroacetic acid and lyophilized. The lyophilisates were dis-

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solved in a radioimmunoassay (RIA) buffer and submitted to RIAs for AM. Intracellular IR-AM contents were measured after extraction and concentration [2]. The viability of THP-1 and HL-60 cells after 40 h incubation was more than 90% by trypan blue staining.

2.4. RIAs for AM

Details of preparation and characterization of antiserum #172CI-7 against human AM (40–52), which can also recognize the whole molecule of AM at the same affinity, have been reported by Sakata et al. [12]. Monoiodinated *N*-Tyr-AM (40–52) isolated by reverse phase HPLC was used as a tracer [2].

2.5. MO-1 antigen expression and DiI-acLDL uptake

After washing three times with cold phosphate-buffered saline (PBS), THP-1 and HL-60 cells were labeled for 10 min at room temperature with fluorescein isothiocyanate (FITC)-labeled MO-1 (CD11b) antibody (Coulter Immunology) or FITC-labeled IgM (Bio Source) as control. DiI-acLDL was added to the culture medium of RPMI 1640 at a final concentration of 5 μg/ml, 5 h before collection of cells. The cells were collected, washed twice with cold PBS, and stored in 0.05% paraformaldehyde in saline at 4°C until flow cytometric analysis. Labeled cells were analyzed on a flow cytometer, Cyto ACE-150 (JASCO Corp.), equipped with an argon laser at 488 nm.

2.6. Isolation of peripheral blood granulocytes, lymphocytes, monocytes and monocyte-derived macrophages

Peripheral blood mononuclear cells and polymorphonuclear cells were isolated from heparinized venous blood of healthy volunteers using a standard Ficoll/metrizoate gradient (Mono-Poly resolving medium, Dainippon Laboratory Products), and then washed with PBS. The mononuclear leukocyte fraction was resuspended in PBS and seeded on monocyte separating plates (MSP) (Japan Immunoresearch Laboratories Co.) at a concentration of 2×10^7 cells/plate. After 1 h incubation to allow for adherence of monocytes, the plates were washed to remove non-adherent cells. The non-adherent cells (lymphocytes) were resuspended in a RPMI medium supplemented with 10% FCS and incubated at 37°C in a CO_2 incubator for 40 h with or without LPS (100 ng/ml). Monocytes (adherent cells) were stripped with 0.02% EDTA and resuspended in the RPMI medium as mentioned above. After culturing in RPMI 1640 medium containing 40% fresh autologous serum for 5 days, monocytes differentiated into monocyte-derived macrophages [13], and then the medium was replaced and incubated for another 40 h with or without LPS. The polynuclear leukocyte fraction, used as granulocytes, was resuspended in the RPMI medium and cultured in the presence or absence of LPS for 40 h. IR-AM content of the medium was measured as described above.

2.7. Statistical analysis

Statistical analysis of the results were performed with one way analysis of variance (ANOVA), followed by a multiple comparison test (Fisher's test). All data were expressed as the mean ± S.E.M. A level of $P<0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Production and secretion of immunoreactive AM from THP-1 and HL-60 cells

The intracellular level of IR-AM was constant and less than 10% of that in culture medium after 40 h incubation in the absence of stimulants (data not shown). This result indicates that AM synthesized in THP-1 and HL-60 cells is not stored in the cells but is secreted constitutively into the medium after synthesis. Thus, we measured IR-AM content in the culture medium after 40 h incubation with various substances to evaluate AM production in THP-1 and HL-60 cells. The basal secretion levels of IR-AM from THP-1 and HL-60 cells were determined to be 0.041 ± 0.005 and 0.056 ± 0.003 fmol/10^5 cells/40 h, respectively (Fig. 1), and their secretion levels were much lower than that from VSMCs and ECs [3–5].

Next, we investigated the effects of substances which activate or differentiate monocyte/macrophage on IR-AM secretion from THP-1 and HL-60 cells [9]. The secretion rate of IR-AM from THP-1 and HL-60 cells stimulated with TPA (10^{-8} M) increased to 0.452 ± 0.013 and 4.535 ± 0.071 fmol/10^5 cells/40 h, respectively. In particular, the secretion rate from HL-60 cells is comparable to that of cultured VSMCs [5]. RA and IFN-γ showed no effect on IR-AM secretion from THP-1 and HL-60 cells (data not shown). LPS increased IR-AM secretion two-fold (0.130 ± 0.002 fmol/10^5 cells/40 h) from HL-60 cells, but not from THP-1 cells. The effects of TNF-α and IL-1β were also examined, since these substances augmented IR-AM secretion from ECs and VSMCs [4,5]. TNF-α augmented IR-AM secretion about six-fold from HL-60 cells (0.355 ± 0.015 fmol/10^5 cells/40 h), but not from THP-1 cells. IL-1β showed no effects on IR-AM secretion from both cell lines (data not shown).

3.2. IR-AM secretion is correlated with differentiation in THP-1 and HL-60 cells

THP-1 is a monocytic leukemia cell line and is known to differentiate into macrophage-like cells, having properties similar to native monocyte-derived macrophages, after incubating with phorbol ester [9]. HL-60 is a promyelocytic leukemia cell line which also differentiates into the macrophage-like cell after treatment with phorbol ester [13].

As we have shown that the AM secretion from THP-1 and HL-60 cells greatly increases after stimulation with phorbol ester (TPA), the relation between AM production and differentiation of the leukemia cell lines was evaluated by measuring the expression of MO-1 antigen under the stimulation of TPA. MO-1 (CD11b), the complement C3b receptor of myeloid cells, is expressed at low levels on myeloblasts, and the cell surface expression of MO-1 has been reported to increase dramatically according to monocyte/macrophage or granulocyte differentiation [14,15]. Thus, the percentage of MO-1 positive cells has been known to be usable as a marker of differentiation. As shown in Fig. 1, the AM secretion was

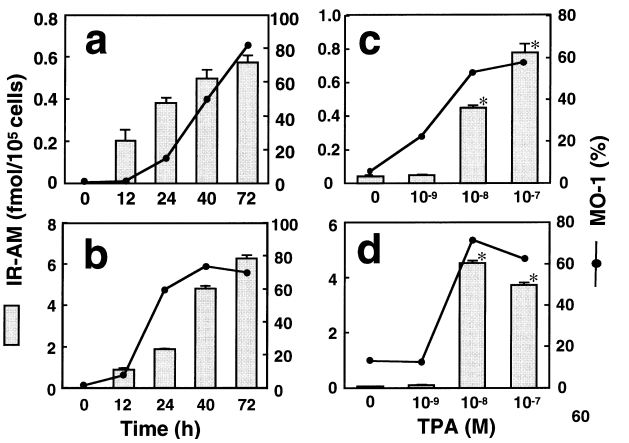

Fig. 1. AM secretion and MO-1 expression on THP-1 cells and HL-60 cells stimulated with TPA. a,b: Time course of AM secretion and MO-1 expression on THP-1 cells (a) and HL-60 cells (b) stimulated with 10^{-8} M of TPA for 0–72 h. c,d: Dose response of AM secretion and MO-1 expression on THP-1 cells (c) and HL-60 cells (d) stimulated for 40 h with TPA at increasing doses indicated. Stippled bar: IR-AM concentration in culture medium of THP-1 cells or HL-60 cells. Closed circle: Positive population of MO-1 expression. Each point represents the mean ± S.E.M. of four separate dishes. *$P<0.05$ vs. control.

elevated according to the increased expression population of the MO-1 antigen on THP-1 and HL-60 cells in a time- and dose-dependent manner. Furthermore, the cells were incubated with TPA (10^{-8} M) for 3 days to evaluate the basal secretion levels of AM from fully differentiated cells. After THP-1 cells were fully differentiated, the resulting THP-1 cells were found to keep secreting IR-AM at a high rate without TPA stimulation (0.784 ± 0.019 fmol/10^5 cells/40 h). In contrast, dexamethasone (10^{-6} M), which antagonizes differentiation into macrophages [13,16,17], markedly reduced IR-AM secretion from THP-1 and HL-60 cells stimulated with 10^{-8} M of TPA to 52.4% and 33%, respectively, although dexamethasone itself did not alter basal secretion of IR-AM (data not shown).

These data indicate that the elevated AM production and secretion from leukemia cell lines is a result of their differentiation into macrophage-like cells rather than the direct effect of TPA on these cell lines.

3.3. Synergistic effects of RA with other stimulants on IR-AM secretion from THP-1 and HL-60 cells

To evaluate cooperative effects of stimulators, IR-AM contents in the culture medium of THP-1 and HL-60 cells were measured after 40 h incubation with all possible combinations of pairs of the six compounds mentioned above. Among them, RA was shown to elicit synergistic effects on IR-AM secretion from THP-1 cells when administered with TNF-α, LPS, IFN-γ or TPA, elevating IR-AM concentrations 3–7-fold compared with the absence of RA (Fig. 2). The other combinations did not induce either additive or synergistic effect on AM production (data not shown). Synergistic effects of RA with TPA, IFN-γ and TNF-α have been widely observed not only in monocytes/macrophages but also in fibroblasts, thyroid carcinoma cell lines and breast cancer cell lines [18–20]. Since RA was shown to differentiate THP-1 cells into cells with macrophage-like characteristics that were slightly different from those of cells differentiated by TPA stimulation [21], the THP-1 cells differentiated with RA were deduced to produce and secrete AM at a higher rate in response to an additional stimulator.

When RA and TPA are coadministered to THP-1 and HL-60 cells, IR-AM secretion from these cells was markedly enhanced (Fig. 2). RA has also been reported to activate macrophages, potentiating phagocytosis and IL-1 activity in macrophage cell line, RAW 264.7 [22]. Based on these data, we deduce that THP-1 and HL-60 cells differentiate into macrophage-like cells in the presence of TPA, and that RA further stimulates AM production and secretion from the resulting macrophage-like cells.

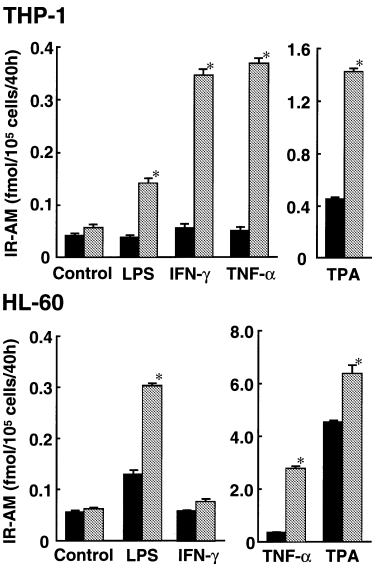

Fig. 2. Effects of coadministration of RA with LPS, IFN-γ, TNF-α or TPA on AM secretion from THP-1 cells and HL-60 cells. THP-1 cells (upper) and HL-60 cells (lower) were incubated separately for 40 h with LPS (100 ng/ml), IFN-γ (100 ng/ml), TNF-α (10 ng/ml), or TPA (10^{-8} M) in the absence (filled bar) or presence (stippled bar) of RA (10^{-6} M). Each value represents the mean ± S.E.M. of four separate dishes. $^*P < 0.05$, compared with IR-AM level in the absence of RA.

On the other hand, RA has been reported to differentiated HL-60 cells into granulocyte-like cells and to increase gene transcription of cytokines such as IL-1, TNF-α and IL-6 [11]. RA (10^{-6} M) synergistically increased IR-AM secretion from HL-60 cells when administered with LPS or TNF-α (Fig. 2). Thus, granulocyte-like cells are thought to have a potential to produce and secrete AM into the circulating blood in the case of sepsis.

As for another mechanism for the synergism in AM production, coadministration of RA with TPA or IFN-γ are reported to increase the expression retinoid receptors in U937 cells and breast cancer cell lines [20,23]. In the case of neuroblastoma cell line, RA has been shown to increase the number of TNF-α receptor [24]. Based on these results, the synergistic effects of RA with TPA, IFN-γ and TNF-α are deduced to be induced in part by mutual augmentation of the receptor expression, and AM production in THP-1 and HL-60 cells is also expected to be synergistically increased by the similar mechanism.

Table 1
AM secretion from peripheral blood granulocytes, lympocytes, monocytes and monocyte-derived macrophages

	IR-AM (fmol/10^5 cells/40 h)	
	Control	LPS 100 ng/ml
Granulocytes	0.118 ± 0.004	0.119 ± 0.015
Lymphocytes	0.158 ± 0.026	0.153 ± 0.009
Monocytes	0.340 ± 0.003*	0.377 ± 0.006
Monocyte-derived macrophages	1.530 ± 0.101**	10.837 ± 0.295***

Peripheral blood granulocytes, lymphocytes, monocytes and monocyte-derived macrophages were cultured in the absence or presence of LPS for 40 h, and the IR-AM content in the culture medium was measured. Each value represents the mean ± S.E.M. of three separate dishes.
$^*P < 0.05$ vs. granulocytes or lymphocytes without LPS. $^{**}P < 0.01$ vs. monocytes without LPS. $^{***}P < 0.01$ vs. monocyte-derived macrophages without LPS.

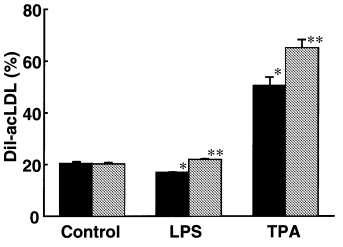

Fig. 3. Increased scavenger receptor activity by AM stimulation on THP-1 cells. Scavenger receptor activity was evaluated as percentage of DiI-LDL positive cells. THP-1 cells were incubated for 40 h with 10^{-7} M of AM (stippled bar) or without AM (filled bar) in the presence of LPS (100 ng/ml) or TPA (10^{-8} M). Each value represents the mean ± S.E.M. of four separate dishes. $*P < 0.05$ vs. control without AM. $**P < 0.05$ vs. without AM in each pair.

3.4. IR-AM secretion from peripheral blood granulocytes, lymphocytes, monocytes and monocyte-derived macrophages

Granulocytes, lymphocytes and monocytes isolated from peripheral blood of healthy volunteers were also shown to secrete IR-AM into culture media (Table 1). Monocytes secreted IR-AM at a rate more than twice that of granulocytes or lymphocytes. Since peripheral blood monocytes were differentiated into monocyte-derived macrophages after culturing in the medium containing 40% fresh autologous serum for 5 days [13], we measured IR-AM concentration in the culture medium from the monocyte-derived macrophages. The monocyte-derived macrophages thus prepared secreted IR-AM at a rate five times higher than that of the monocytes, and the AM secretion was further augmented about seven-fold by LPS stimulation (Table 1). Thus, AM is found to be produced and secreted from lymphocytes, granulocytes, monocytes and monocyte-derived macrophages isolated from peripheral blood. AM secretion from the monocyte-derived macrophages are further increased by LPS stimulation as in the cases of THP-1 and HL-60 cells differentiated by RA stimulation.

3.5. AM increase the scavenger receptor activity on THP-1 cells

Hampton et al. have indicated that macrophage scavenger receptors scavenge LPS as well as modified LDL and reduce LPS in the circulation or tissues [25]. To evaluate the function of AM in septic pathophysiology, we measured the scavenger receptor activity by specific receptor-mediated uptake of DiI-acLDL, a fluorescent-labeled acLDL probe [26]. TPA treatment on THP-1 cells has been shown to increase the scavenger receptor activity according to their differentiation into macrophage-like cells [14,25,27]. We confirmed that the scavenger receptor activity was augmented after 40 h stimulation with TPA, while LPS significantly reduced the scavenger receptor activity (Fig. 3). The scavenger receptor activity on monocyte-derived macrophages is also reported to be suppressed by LPS [28]. Mice lacking the scavenger receptor gene are more susceptible to endotoxin shock, and the serum levels of TNF-α and IL-6 are higher in mice lacking the scavenger receptor gene than those in the wild type [29]. These data indicate that the suppression of scavenger receptor activity by LPS decreases the clearance of LPS, augments the production of proinflammatory cytokines, and decreases the survival rate of endotoxin shock. Thus, the scavenger receptor activity is one of the important factor for modulating the severity of sepsis. As shown in Fig. 3, AM increased the scavenger receptor activity on THP-1 cells stimulated with TPA or LPS, although AM itself showed no effect on the activity. These data suggest the possibility that AM reduces the effect of LPS by increasing the scavenger receptor activity on monocytes/macrophages.

We have reported that the synthesis and secretion of AM from VSMCs and ECs are augmented by LPS, TNF-α and IL-1β [4,5], and that intravenous administration of LPS into rats markedly increases plasma AM concentration and AM gene transcription in almost all tissues [6]. Furthermore, the average concentration of plasma AM in patients with septic shock has recently been reported to be 45 times higher than that of healthy volunteers [7]. Based on these data, we have attributed high plasma AM concentrations in septic shock to the elevated production of AM in VSMCs and ECs. In this study, we demonstrated that THP-1 and HL-60 cells produced and secreted AM according to their differentiation into macrophages. We also showed that LPS and TNF-α significantly increased AM production and secretion from HL-60 cells. The effects of LPS and TNF-α on AM secretion from THP-1 and HL-60 cells were greatly enhanced in the presence of RA (Fig. 2). Even with monocyte-derived macrophages from peripheral blood, LPS highly augmented AM secretion (Table 1). Taken together, these data demonstrate that monocytes/macrophages are one of the sources of plasma AM especially in the case of sepsis. In addition, the present study provides evidence that AM secreted from monocytes/macrophages increases the scavenger receptor activity on themselves.

In conclusion, monocytes/macrophages should be recognized as one of the major sources of AM in the circulating blood, and the secreted AM may participate in the hypotension as well as regulation of macrophage functions in the septic pathophysiology.

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