Preactivated Peripheral Blood Monocytes in Patients With Essential Hypertension

Yvonne Dörffel, Christoph Lätsch, Bruno Stuhlmüller, Stefan Schreiber, Susann Scholze, Gerd R. Burmester, Jürgen Scholze

Abstract—The purpose of this study was to investigate the possible involvement of human peripheral blood monocytes in the pathology of hypertensive disease. We determined the in vitro secretion patterns of proinflammatory cytokines obtained from isolated peripheral monocytes from normal controls and from hypertensive patients either after in vitro stimulation with angiotensin II (Ang II) with or without preincubation with an Ang II type 1 receptor antagonist (losartan) or after stimulation with lipopolysaccharide. Blood samples were obtained from 22 patients with essential hypertension (before any drug administration or after interruption of antihypertensive therapy) and from 24 normotensive healthy individuals used as a control group. Peripheral blood monocytes were isolated by density gradient centrifugation and plastic adherence. The state of monocyte activity was determined by the capacity to secrete tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6, (IL-6) either spontaneously or after stimulation. Cytokine concentrations were determined in culture supernatants by specific ELISA. Proinflammatory cytokine levels were assessed by semiquantitative reverse transcribed polymerase chain reaction. After stimulation with Ang II, the IL-1β secretion of peripheral blood monocytes was significantly increased in hypertensive patients versus healthy individuals (P<0.05). In contrast, in monocytes preincubated with losartan before exposure to Ang II, IL-1β secretion was diminished in both groups to comparable levels. The secretion of IL-1β and TNF-α was significantly increased in peripheral blood monocytes from hypertensive patients versus healthy individuals after stimulation with lipopolysaccharide (TNF-α, P<0.02; IL-1β, P<0.05). Upregulation of IL-1β and TNF-α secretion in peripheral blood monocytes from hypertensive patients was also seen at the RNA level. Our results indicate preactivated peripheral blood monocytes in hypertensive patients. Ang II may be directly involved in the process of monocyte activation. (Hypertension. 1999;34:113-117.)

Key Words: monocytes, human hypertension, essential tumor necrosis factor interleukins polymerase chain reaction angiotensin II

Immunopathogenic mechanisms may be involved in the pathogenesis of hypertensive disease. Alterations in both humoral and cellular immunity have been described. The possible role of leukocytes in the pathology of hypertension has been a matter of great interest. The available information on monocytes is scarce compared with available information on neutrophils as a result of the difficulty of isolating monocytes from peripheral blood.

Bataillard et al demonstrated that the in vivo administration of silica, a selective toxin to monocytes, reduced the degree of hypertension in Lyon hypertensive rats and inhibited the formation of hypertrophy of the left ventricle. Recent reports indicate that in spontaneously hypertensive rats (SHR) the number of activated monocytes is significantly elevated. McCarron et al showed a significant increase of monocyte adhesion to endothelial cells from SHR after stimulation with lipopolysaccharide (LPS) or proinflammatory cytokines (interferon-γ, interleukin-1β [IL-1β], and tumor necrosis factor-α [TNF-α]) versus normotensive Wistar-Kyoto rats.

Whereas McCarron proposed that the secretion of endothelial cell factors was most likely to have caused the monocyte activation, we investigated whether the peripheral blood monocytes from patients with essential hypertension are already preactivated by angiotensin II (Ang II). IL-1β, TNF-α, and interleukin-6 (IL-6) are proinflammatory cytokines produced principally by activated monocytes or macrophages. The increased secretion of these cytokines is considered to be an activation marker of circulating monocytes. We analyzed the activation status of circulating monocytes from patients suffering from essential hypertension versus normal controls after stimulation with Ang II in

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physiologically relevant concentration or with LPS, which typically stimulates monocytes.7

Methods

Chemicals
Endotoxin-free fetal calf sera (endotoxin < 0.002 ng/mL) were purchased from Biochrom KG/Seromed. Losartan was obtained from Merck&Co, Inc. Cytokine assays were obtained from R&D Systems (IL-1β, IL-6) or from Medgenix Diagnostics (TNF-α). If not otherwise specified, all other chemicals were purchased from Sigma Chemical Co.

Patients
Men (n = 13) and women (n = 9) outpatients, aged 31 to 75 years (mean age 46 years), with mild to moderate essential hypertension (measured before any drug administration or after they had discontinued all antihypertensive medications for ≥10 days) participated in the study. Mean body weight was 78 ± 13 kg and height was 1.72 ± 0.03 m. All patients underwent routine 24-hour ambulatory blood pressure (BP) measurement for clinical evaluation. Only patients with a sitting diastolic BP between 95 and 114 mm Hg were included. Exclusion criteria included all other diseases and abnormal laboratory tests results (levels of serum potassium, creatinine, liver enzymes, erythrocyte sedimentation rate, C-reactive protein, white blood cells, thrombocytes). Patients were also excluded from the trial if they had severe or secondary hypertension. Informed consent was obtained from all patients. The study was granted prior approval by the local ethics review committee. Thus, the procedures followed were in accordance with the institutional guidelines of Humboldt University.

Human Peripheral Blood Monocytes

Isolation
Peripheral blood mononuclear cells were isolated as described previously.8 Peripheral blood diluted with PBS containing 100 U heparin per milliliter was layered over Ficoll-Histopaque (Pharmacia AB, specific gravity: 1.077) and centrifuged for 40 minutes at 400g. Cells harvested from the interface were washed in HBSS (without Ca2+ or Mg2+) and resuspended in RPMI 1640 with 10% fetal calf serum (FCS). Cells were layered on Petri dishes and incubated for 1 hour (37°C). Nonadherent cells were discarded from the petri dishes by thorough rinsing with prewarmed RPMI 1640 (37°C). More than 99% of the adherent cells were collected by rinsing with cold (4°C) HBSS and by gentle mechanical scraping. The adherent cells were washed in HBSS and resuspended in RPMI 1640 (with 10% FCS, penicillin 100 U/mL, and streptomycin 100 mg/mL) for cell culture. Viability was > 98%, as determined by trypan blue exclusion after isolation of the peripheral blood monocytes. Cell viability was also determined by the propidium iodide-positive staining method.9 The proportion of propidium iodide–positive cells was consistently < 5%.

Identification of Peripheral Blood Monocytes: Fluorescence-Activated Cell Sorter
Monocytes were identified using immunofluorescence staining for cell surface antigens. Monoclonal mouse antibodies specific for human T cells (CD3; Clone UCHT1, code F0818), human B cells (CD19; Clone HD37, code F0768), and human monocytes (CD14; Clone TÜK4, code R0864) were obtained from DAKO A/S. An appropriate control using antibodies with an irrelevant specificity was run to determine nonspecific staining. The cells stained with antibodies were analyzed with a fluorescence activated cell sorter (FACScalibur, Becton Dickinson Immunochemistry Systems). The relative frequency of cells expressing the various cell surface antigens was calculated after computer subtraction of the control from the experimental fluorescence profiles. The cell suspension contained 93% monocytes.

Stimulation of Peripheral Blood Monocytes and Monocyte Culture
The optimal conditions and concentration for stimulation with Ang II and LPS were determined by time and concentration kinetics. The cytokine levels increased during stimulation with Ang II or LPS over a 24-hour period.

Peripheral blood monocytes were cultured at a concentration of 105 cells/mL in RPMI 1640 (supplemented with 10% FCS, 1% pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µg/mL gentamicin). Cells were cultured in 24-well plates (Nunc, Roskilde) for 24 hours with or without a physiologically relevant concentration of Ang II (10−8 mol/L)10 or LPS (10 ng/mL). In additional experiments, monocytes were preincubated with losartan (10−8 mol/L) for 30 minutes. After 24 hours, supernatants were separated from cells by centrifugation, snap-frozen, and stored at −70°C until cytokine levels were determined, usually within 2 weeks after culture.

Cytokine Assays
Supernatant concentrations of IL-1β and IL-6 were assessed using a specific sandwich ELISA by R&D Systems. TNF-α was determined with a specific ELISA manufactured by Medgenix. All samples were analyzed in triplicate. The amount of cross-reactivity was assessed by comparison with the concentration yielding a 50% inhibition of binding. Sensitivity levels were between 0.3 (IL-1β) and 3.0 pg/mL (TNF-α); intra-assay and inter-assay precision variability was < 3% (controls, data not shown).11 ELISA antibodies against TNF-α, IL-6, and IL-1β, respectively, were used to absorb the cytokines from cell culture supernatants.

Total RNA Extraction
Total RNA was isolated using a commercial kit (RNAeasy, Qiagen GmbH) with minor modifications after 6 hours as described by Chomczynski et al.12 Subsequently, RNA was extracted from the aqueous phase and mixed with ethanol using Qiagen columns. Total amount and purity of RNA were determined by spectrophotometry and gel electrophoresis procedure (1% agarose).

Reverse Transcription
Single-stranded cDNA was transcribed from 3 µg of total RNA with 500 ng oligo(dT)12-18 primer (Gibco-BRL, Life Technologies GmbH) in a volume of 20 µL containing 50 mmol/L Tris-HCl, 75 mmol/L KCl, 3 mmol/L MgCl2, 10 mmol/L DTT, and 1 mmol/L of each dNTP, as well as 200 U Superscript II reverse-transcriptase (Gibco-BRL). The reaction mixture was incubated at 42°C for 1 hour to generate cDNA.

Semiquantitative Polymerase Chain Reaction
Two µL of the cDNA sample (corresponding to ~300 ng of total RNA) was added to a mixture of 10 µL polymerase chain reaction (PCR) buffer (200 mmol/L Tris-HCl [pH 8.4], 500 mmol/L KCl), 3 µL MgCl2 (50 mmol/L), 2 µL dNTP (10 mmol/L), 1 µL each of 5' and 3' primer (25 µmol/L), 5 U Tag DNA polymerase, and 80 µL autoclaved, distilled water. The following primer pairs (Perkin Elmer) were used: IL-1β (802 bp), 5' primer ATG GCA GAA GTA CCT AAG TGC GC, 3' primer ACA CAA ATT GTA TGG TGC TT; TNF-α (945 bp), 5' primer GGC CCA GGC AGT CAG AT, 3' primer CAC AAG TGC AAA CAT AAA TAG AGG; and GAPDH (422 bp), 5' primer GTG CAT CCA TGA TCA CTT TG, 3' primer CTG GTT GAG GGC AAT GCC.

PCR conditions were as follows: 94°C for 45 seconds, 62°C for 1 minute, and 72°C for 1 minute. Finally, extension was performed for 10 minutes at 72°C. During the amplification period aliquots of the reverse transcription (RT)-PCR products were taken from each tube at cycles 25, 28, 30, 35, and 40 to determine the linear increase of amplification. Calculation of peak areas was based accordingly on cycle 30. Five µL of the amplification products of the same cycle mixed with loading buffer (containing 0.25% bromphenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water) was loaded with 4 µL of Low DNA Mass ladder (Gibco-BRL) on a 1% agarose
Results

Spontaneous secretion of IL-1β (patients, 78±21 pg/mL; controls, 49±9 pg/mL), TNF-α (patients, 113±16 pg/mL; controls, 104±12 pg/mL), and IL-6 (patients, 68±8 pg/mL; controls, 63±11 pg/mL) into culture supernatants (Figure 2). The secretion of IL-1β (patients, 2337±337 pg/mL; controls, 1458±165 pg/mL; P<0.05) and TNF-α (patients, 4371±727 pg/mL; controls, 380±318 pg/mL; P<0.02) was significantly increased in peripheral blood monocytes derived from patients with essential hypertension after stimulation with LPS versus normal controls. In contrast, the secretion of IL-6 did not differ between groups (patients, 4587±668 pg/mL; controls, 4969±437 pg/mL).

Statistical Analysis of the Density Scans Adjusted to Internal Standard GAPDH of the Semiquantitative RT-PCR Products Using Specific Primers for IL-1β and TNF-α From Monocytes of Hypertensive and Normotensive Patients

<table>
<thead>
<tr>
<th>RT-PCR Product Groups</th>
<th>IL-1β</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated controls</td>
<td>835±278</td>
<td>381±127</td>
</tr>
<tr>
<td>LPS-stimulated controls</td>
<td>2500±419</td>
<td>444±148</td>
</tr>
<tr>
<td>Unstimulated patients</td>
<td>2088±926</td>
<td>1178±933</td>
</tr>
<tr>
<td>LPS stimulated patients</td>
<td>4170±672*</td>
<td>2710±692</td>
</tr>
</tbody>
</table>

Values represent mean±SEM of peak area. *P<0.05 vs LPS-stimulated controls.

ment, the IL-1β secretion was diminished in both groups to comparable levels that did not differ statistically (patients, 389±255 pg/mL; controls, 112±82 pg/mL; Figure 1). The secretion of TNF-α and IL-6 did not differ between groups.

Cytokine Assays-Response to LPS Stimulation

The activation of peripheral blood monocytes was monitored by measuring the LPS-induced release of IL-1β, IL-6, and TNF-α into culture supernatants (Figure 2). The secretion of IL-1β (patients, 2337±337 pg/mL; controls, 1458±165 pg/mL; P<0.05) and TNF-α (patients, 4371±727 pg/mL; controls, 380±318 pg/mL; P<0.02) was significantly increased in peripheral blood monocytes derived from patients with essential hypertension after stimulation with LPS versus normal controls. In contrast, the secretion of IL-6 did not differ between groups (patients, 4587±668 pg/mL; controls, 4969±437 pg/mL).

Semiquantitative RT-PCR Results

To study whether the increase of IL-1β and TNF-α production in peripheral blood monocytes of hypertensive patients was due to a transcriptional upregulation, we performed a semiquantitative RT-PCR. A significant elevation of LPS-induced secretion from proinflammatory cytokines in patients versus controls could be seen with both IL-1β and TNF-α amplification products of total RNA (P<0.05). This paralleled the findings seen on the protein level. The Table shows results of density-scan measurements of the RT-PCR products for patients and control group. After stimulation with Ang II, a difference between patients and controls could be seen only with the IL-1β amplification products of total RNA (data not illustrated).
cytes also show an elevated activation status. This emphasizes the importance of inflammatory mechanisms in inflammatory reactions. Most of the studies concentrate on tissue activated monocytes or macrophages are mediators of inflammatory reactions such as atherosclerosis. It has been shown that, in hypertension, endothelial dysfunction and subendothelial accumulation of monocytes occurs. It is generally hypothesized that hypertension may enhance the responsiveness of the endothelium to factors that promote monocyte adhesion. The endothelial cells may be involved in the activation of peripheral blood monocytes. Some of these findings, however, may be mediated by the increased production of TNF-α and IL-1β in peripheral blood monocytes derived from patients with essential hypertension. TNF-α, IL-1β, and IL-6 produced by activated monocytes or macrophages are mediators of inflammatory reactions. Most of the studies concentrate on tissue macrophages, which transform into foam cells. These studies emphasize the importance of inflammatory mechanisms in atherosclerosis. We demonstrate here that circulating monocytes also show an elevated activation status.

To the best of our knowledge, this is the first report about preactivated circulating human monocytes in essential hypertension. Similar results were obtained in hypertensive rats by several groups. The question might arise as to whether preactivation of the monocytes is related to the isolation method. We are not aware of any method of monocyte isolation without at least a low possibility of activation, especially when small amounts of blood are processed. However, possible monocyte activation during the isolation process appears to be of minor importance; we compared monocytes of hypertensive patients with those of normal controls whose monocytes were isolated in the same manner and detected significant differences between groups. Until now, we could not exclude the possibility that in circulation only subgroups of monocytes may appear.

This investigation showed elevated TNF-α and IL-1β levels secreted from peripheral blood monocytes derived from hypertensive patients versus control subjects after stimulation with LPS. We used endotoxin LPS to investigate the mechanism of monocyte activation. Monocyte stimulation by LPS is a long-established method of testing the maximum degree of activation of these cells. LPS stimulation of monocytes induces TNF-α production, which subsequently triggers synthesis of IL-1β and IL-6. Increased IL-1β and TNF-α secretions are associated with significantly increased RNA expressions observed in hypertensive patients.

These cytokines cause alterations of endothelial cells in vivo and increase neutrophil adherence to the endothelium by enhancing the expression of intercellular adhesion molecule-1. Komatsu and colleagues showed that the differences induced in intercellular adhesion molecule-1 expression between SHR and Wistar-Kyoto rats did not appear to be due to differences in endothelial surface areas. They suspected an abnormal inflammatory response associated with the hypertensive disease. The possibility exists that elevated levels of circulating cell adhesion molecules in essential hypertension are due to activated monocytes. It was demonstrated that TNF-α exerts a direct toxic effect on cultured vascular endothelial cells and induces the apoptosis of endothelial cells. In this regard, the observations of Bevilacqua et al are of note. They found that TNF and IL-1 induce procoagulant activity in cultured human vascular endothelium. Therefore, IL-1β and TNF-α may play a role in the development of atherosclerosis. Wick et al suggest that an autoimmune reaction against heat-shock protein 60, expressed by endothelial cells after pretreatment with certain cytokines (TNF-α, IL-1β), is the initiating event in atherogenesis. Furthermore, the upregulation of the transforming growth factor-β system in monocytes of hypertensive patients supports the view of monocyte involvement in the pathogenesis of atherosclerotic lesions associated with hypertension. None of our patients showed elevated IL-6 values after stimulation with LPS of the peripheral blood monocytes, which parallels normal laboratory parameters such as C-reactive protein and leukocyte counts.

It is not clear from this study whether preactivated monocytes in hypertensive patients are an epiphenomenon or a causal factor triggering hypertension and/or atherosclerosis. In further studies, the monocyte–endothelial cell interaction of these patients will have to be investigated. In theory, the elevated proinflammatory cytokine levels may also be caused by arteriosclerotic lesions; we observed a slight relationship between age and IL-1β values, which correspond to an increasing risk of atherogenesis with age. In our study, we excluded patients with arteriosclerotic lesions of the carotid artery and abdominal aorta.

**Correlation Analysis**

To evaluate whether hypertension influences the determined values or whether cytokine levels depend more on age and/or gender, we correlated BP, age, and gender of the study population with the cytokine levels. Correlation was investigated for IL-1β, IL-6, and TNF-α after stimulation with LPS. There was no statistically relevant relation between age and values of IL-1β but a slight correlation with age (r=0.46; P<0.002) and systolic BP (r=0.48; P<0.001) (Figure 3). Similar results were obtained for TNF-α (age, r=0.35; P<0.05; systolic BP, r=0.47, P<0.002). We found no correlation for IL-6.

**Discussion**

Hypertension is commonly associated with the development of atherosclerosis. It has been shown that, in hypertension, endothelial dysfunction and subendothelial accumulation of monocytes occurs. It is generally hypothesized that hypertension may enhance the responsiveness of the endothelium to factors that promote monocyte adhesion. The endothelial cells may be involved in the activation of peripheral blood monocytes. Some of these findings, however, may be mediated by the increased production of TNF-α and IL-1β in peripheral blood monocytes derived from patients with essential hypertension. TNF-α, IL-1β, and IL-6 produced by activated monocytes or macrophages are mediators of inflammatory reactions. Most of the studies concentrate on tissue macrophages, which transform into foam cells. These studies emphasize the importance of inflammatory mechanisms in atherosclerosis. We demonstrate here that circulating monocytes also show an elevated activation status.

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The relationship between IL-1β levels and BP supports the possibility that high BP itself preactivates the peripheral blood monocytes. Because monocytes in circulation are in a low-pressure region most of the time, it is unlikely that high BP itself preactivates the peripheral blood monocytes.

To investigate the relevance of monocyte activation in clinical practice, we studied monocyte activity after stimulation with Ang II at physiologically relevant concentrations. It is well documented that Ang II reduces blood flow, modulates vascular remodeling, leads to incremental protein synthesis in vascular smooth muscle cells, and increases the synthesis of collagen type I and III in fibroblasts, which leads to thickening of the vascular wall. We were able to demonstrate that monocytes of hypertensive patients are preactivated not only after LPS stimulation but also after Ang II stimulation, which results in increased secretion of IL-1β in hypertensive patients. Such changes in hypertensive patients versus healthy controls were also seen at the RNA level.

In the present study, Ang II triggered IL-1β production without triggering production of TNF-α. This finding suggests that the Ang II signal-transduction mechanism in monocytes may be different from the mechanism of endotoxin. While nuclear factor-kB transcription factor plays a major role in LPS-mediated production of TNF-α by monocytes, alternative elements seem to regulate IL-1β gene induction after Ang II stimulation.

Ongoing discussions are taking place in many articles regarding the inflammatory genesis of atherosclerosis and the role of proinflammatory cytokines. In our opinion, Ang II–preactivated circulating monocytes in hypertensive patients may lead to subendothelial infiltration and subsequently enhance the risk of arteriosclerotic complications. Prevention of Ang II–mediated monocyte activation by losartan (Figure 1) may be a novel therapeutic approach to the question, among others, of whether elevated Ang II levels preactivate the peripheral blood monocytes.

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References

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