Broadly Altered Gene Expression in Blood Leukocytes in Essential Hypertension Is Absent During Treatment

Helena Chon, Carlo A.J.M. Gaillard, Brenda B. van der Meijden, Hilde M. Dijstelbloem, Rob J. Kraaijenhagen, Dik van Leenen, Frank C.P. Holstege, Jaap A. Joles, Hans A.R. Bluysen, Hein A. Koomans, Branko Braam

Abstract—We assessed whether large-scale expression profiling of leukocytes of patients with essential hypertension reflects characteristics of systemic disease and whether such changes are responsive to antihypertensive therapy. Total RNA from leukocytes were obtained from untreated (n=6) and treated (n=6) hypertensive patients without apparent end-organ damage and from normotensive controls (n=9). RNA was reverse-transcribed and labeled and gene expression analyzed using a 19-K oligonucleotide microarray using dye swaps. Samples of untreated and of treated patients were pooled for each sex and compared with age- and sex-matched controls. In untreated patients, 680 genes were differentially regulated (314 up and 366 down). In the treated patients, these changes were virtually absent (4 genes up, 3 genes down). A myriad of changes was observed in pathways involved in inflammation. Inflammation-dampening interleukin receptors were decreased in expression. Intriguingly, inhibitors of cytokine signaling (the PIAS family of proteins) were differentially expressed. The expression of several genes that are involved in regulation of blood pressure were also differentially expressed: angiotensin II type 1 receptor, ANP-A receptor, endothelin-2, and 3 of the serotonin receptors were increased, whereas endothelin-converting enzyme-1 was decreased. Strikingly, virtually no changes in gene expression could be detected in hypertensive patients who had become normotensive with treatment. This observation substantiates the long-standing idea that hypertension is associated with a complex systemic response involving inflammation-related genes. Furthermore, leukocytes display differential gene expression that is of importance in blood pressure control. Importantly, treatment of blood pressure to normal values can virtually correct such disturbances. (Hypertension. 2004;43:1-5.)

Key Words: hypertension, essential ■ renin angiotensin system ■ gene expression ■ leukocytes

In Western societies, the prevalence of hypertension is ~10% and forms a major risk factor for atherosclerosis. Antihypertensive treatment can reduce cardiovascular risk; however, it does not normalize it. At present, we are not able to identify these patients who, despite normalization of blood pressure, are still at risk for atherosclerotic complications. There is no doubt that atherosclerosis is a multifaceted and complex systemic disease, involving cells of the vascular wall, circulating cells, and a variety of neurohumoral factors. The initiation of disruption of vascular wall integrity is characterized by endothelial cell dysfunction and a strong relation with systemic inflammation is suspected. Elevated C-reactive protein, interleukin (IL)-6, and soluble intercellular adhesion molecule (ICAM)-1 are all associated with increased cardiovascular risk.1 Recently, Yasunari et al have demonstrated a relationship between oxidative stress in polymorphonuclear leukocytes and mononuclear cells and C-reactive protein values in hypertensive patients.2

Expression profiling offers novel possibilities to classify and monitor disease and to follow treatment, particularly when this can be achieved on easily available material. As such, hypertension can be interesting, because it forms a highly prevalent prologue for atherosclerosis and forms a diagnostic dilemma, because normalization of blood pressure is not necessarily achieved by treating the causal agent and does not necessarily reduce cardiovascular risk to normal.

We investigated whether the expression profile in leukocytes of hypertensive patients already reflects such a systemic inflammation. Leukocytes in a hypertensive environment are subjected to increased shear-stress during their voyage in the circulation.3 In addition, they are probably also sensitive to increased oxidative stress by the vascular wall and may respond to primary driving forces of hypertension, such as angiotensin II.4 Although a response to such factors is probably not the primary function of leukocytes, it is likely that they could alter leukocyte gene expression. We wondered whether leukocytes could be used as “biosensors” of a change in their environment. Because hypertension is reversible, we also investigated whether such disturbance is corrected during treatment.
Methods

An expanded Methods section is available online at http://www.hypertensionaha.org.

Patients Selection

In our hypertension screening facility, patients with hypertension are assessed for potential secondary causes, the severity of hypertension, other risk factors, and end-organ damage. For the present study, the inclusion criterion was untreated (whole-day average >140/85) or treated hypertension (whole-day average <140/85) as determined by 24-hour blood pressure assessment. Exclusion criteria were diabetes, smoking, renal failure (creatinine clearance <70 mL/min), and end-organ damage (coronary heart disease, stroke, peripheral artery disease, microalbuminuria). Age-matched nonsmoking normotensive subjects devoid of end-organ damage served as control. The protocol was approved by the Medical Ethical Committee of the University Medical Center and informed consent was obtained from each subject.

Collection of Blood Samples and RNA Extraction

Thirty milliliters of whole blood was drawn in ice-cold EDTA-containing tubes and erythrocytes removed using a hypotonic lysis buffer. Trizol reagent (Invitrogen, Carlsbad, Calif) was added to the collected suspension and immediately stored at −80°C until further processing. RNA was isolated according to the manufacturer’s instructions, quantitated using a spectrophotometer (Shimadzu Scientific Instruments, Columbia, Md), and stored at −80°C until further processing. Total RNA was isolated and pooled per sex and patient group.

Microarray Experiments

In-house manufactured human 70-mer microarrays containing 19 200 spots were applied.5 Probes were prepared by amino-allyl-incorporated reverse-transcription (Invitrogen, Carlsbad, Calif) and labeled with Cy3 and Cy5 fluorescent dyes (Amersham Biosciences UK Unlimited, Buckinghamshire). For each comparison, a dye-swap was used. Thus, middle-aged untreated males (n = 3) and females (n = 3) and treated males (n = 3) and females (n = 3) were compared with age- and sex-matched controls (n = 3 males and n = 6 females). Cy3-labeled test samples were then mixed with the corresponding Cy5-labeled control sample and vice versa (dye swap approach) for each specific experiment and added to a 2× hybridization buffer and hybridized overnight at 42°C.

Microarray Scanning and Image Analysis

Microarray slides were scanned using Agilent DNA microarray scanner Model G2565BA (Agilent Technologies, Palo Alto, Calif). Stored images were analyzed using Imagene (Biodiscovery, Marina Del Rey, Calif) software. Data were normalized using the Lowess algorithm, as was described by van Peppel et al.5 Only spots with a signal intensity exceeding background +2 SD were considered. Log transformed signal intensities between channels were normalized to have an identical log2 median. For the present report, ratios of both sexes were averaged; only those absolute log2 ratios that exceeded 0.7 (1.6-fold) in both sexes were considered. Functional classification of the genes was obtained using the Gene Ontology Consortium classification of biological processes and by searching public databases. Cluster analysis was performed using the EPclust software developed by Jaak Vilo.7

Data Analysis

Clinical parameters were compared using 1-way ANOVA and the Student Newman Keuls post hoc test (SigmaStat; SPSS, Chicago, Ill); P<0.05 is considered statistically significant.

Reverse-Transcriptase Polymerase Chain Reaction

Pooled RNA per group was reverse-transcribed (Invitrogen Life Sciences, Carlsbad, Calif) and formed cDNA was used in polymerase chain reactions using Taq DNA polymerase (Invitrogen Life Sciences). Polymerase chain reaction products were quantitated on a 2% agarose gel (Roche) with Gel Doc 2000 (BioRad). The primers and conditions used for polymerase chain reaction are summarized (see online Table I at http://www.hypertensionaha.org). Of the 35 genes tested, 15 were confirmed, 6 did not display a signal, 6 were inconsistent, 6 did not show changes, and 2 were opposite to the microarray data.

Results

The Table summarizes the clinical data. Systolic and diastolic blood pressure was significantly elevated in untreated but not in treated patients. Drugs used were angiotensin-converting enzyme (ACE) inhibitors (n = 3), β-blockers (n = 2), or a combination of β-blockade and diuretic (n = 1). Age, body

Clinical Data for Untreated and Treated Hypertensive Subjects and Controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Untreated</th>
<th>Treated</th>
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<tr>
<td>N</td>
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<td>6</td>
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<tr>
<td>Age, y</td>
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<td>BMI</td>
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<td>25±2</td>
<td>26±2</td>
</tr>
<tr>
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<td>146±10*</td>
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<tr>
<td>Diastolic blood pressure, mm Hg</td>
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<tr>
<td>Glucose, mmol/L</td>
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<td>Creatinine, μmol/L</td>
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<td>Creatinine clearance, mL/min</td>
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<td>Microalbuminuria, mg/d</td>
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<td>6±2</td>
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<tr>
<td>Framingham risk score</td>
<td>2.4±6.1</td>
<td>6.3±5.1</td>
<td>4.5±2.6</td>
</tr>
</tbody>
</table>

*Average±SD. P<0.05 versus controls and treated.

Figure 1. Distribution of log2 ratios for all genes binned per 0.05 log2 ratio, displayed for patients with untreated and treated hypertension. The number of genes per bin close to zero (indicating no change) are much higher in the treated group, whereas almost no genes were found in the bins above and below a log2 value of 0.7 (1.6× up) and −0.7 (1.6× down). In contrast, many genes in the untreated group were found in the bins above these levels.
weight, cholesterol, glucose, creatinine, creatinine clearance, or microalbuminuria was not different between groups. All expression data can be found (see online Table II at http://www.hypertensionaha.org). In untreated hypertension patients, 680 genes were differentially regulated (314 up and 366 down). In the treated patients, these changes were virtually absent (4 genes up, 3 genes down). The distribution of log$_2$ ratios is shown in Figure 1. In the samples from untreated patients, the number of genes deviating from a log$_2$ ratio of 0 is substantially higher than in the samples from treated patients. Figure 2 displays the cluster analysis of the individual groups. Genes were selected when 1 of the 4 comparisons had an absolute log$_2$ ratio exceeding 1.0. This analysis reveals that many changes were similar in the untreated male and female patients.

First, profiles were analyzed toward oxidative stress- and inflammation-related differential gene expression. In the untreated patients, the expression of 2 isoforms of the NADPH-oxidase component gp91phox (NOX1 and NOX3 or gp91-2 and gp91-3) and inducible NOS were increased. Decreased expression was found for catalase and superoxide dismutase 1, genes that reduce oxidative stress and for the detoxifying enzyme thioredoxin reductase 1.

A very complex response was observed in expression of inflammation-related genes. A potential driving force for inflammation was formed by the upregulated calcineurin subunit A-α, a stimulator of nuclear factor of activated T cells (NF-AT) and downregulation of calcineurin binding protein 1 (CABIN1). Of the small inducible cytokine (SCY) family, RANTES (SCYA5) was decreased and SCYA18 and SCYB6 were increased. Expression of the interleukin (IL)-4, IL-10A, and IL-13x1 receptors, all involved in dampening of the inflammatory response, was decreased. Both the IL-1R2 (the so-called IL-1 decoy) receptor and the IL-1 receptor accessory protein, both involved in anti-inflammation by opposing the actions of IL-1, tended to be downregulated. Expression of the IL-17 receptor, which mediates a proinflammatory signal, was also decreased. Expression of the signal transducer and activator of transcription factor 3 (STAT3), one of the mediators of cytokine signaling, was diminished. The PIAS family of proteins inhibits the actions of STAT proteins, and 4 members have been identified: PIAS1, PIAS3, PIASx, and PIASy. The expression data indicate differential regulation of 3 of these factors; expression of PIAS1 and PIASy was decreased, and that of PIAS3 was increased. PIASx-β tended to decrease on the array (log$_2$ ratio −0.63) and was decreased in the reverse-transcriptase polymerase chain reaction. In this respect, the increased expression of insulin-like growth factor-1 is remarkable. Insulin-like growth factor-1 is activated by growth hormone (which acts via the JAK/STAT pathway), can activate calcineurin, and can increase the expression of the IL-1R2 receptor. The changes are summarized in Figure 3 and the gels of the reverse-transcriptase polymerase chain reaction for the genes that were confirmed are displayed.

Next, we searched for other function groups that displayed modulation of transcription and were associated with blood pressure regulation or other events in early atherosclerosis. Quite remarkable increases in gene expression were observed in 5-hydroxytryptamine receptors (HTR) HTR1D, HTR2B, HTR2C, HTR3B, and HTR3D.
and HTR3A. Of these, changes in the HTR1D and HTR3A receptor could be confirmed by reverse-transcriptase polymerase chain reaction; we extended the reverse-transcriptase chain reaction with the HTR2A receptor (Figure 4). The expression of monoamine oxidase, which degrades 5-HT, was not changed. It is unclear whether the observed decrease in expression of the aldehyde dehydrogenases ALDH1A3, ALDH7, and ALDH9 is relevant for further serotonin degradation. The expression of the angiotensin AT1 receptor (AGTR1; 2 representations on the chip) was just below the used cut-off; however, reverse-transcriptase polymerase chain reaction analysis of this gene confirmed increased expression (see Figure 4). Genes for endothelin-2 (EDN2) and ANP-A receptor displayed increased expression, whereas endothelin-converting enzyme 1 (ECE-1) was decreased (see Figure 4). The expression of BMP1, and its 1A receptor, genes involved in bone mineralization were both increased. Genes coding for channels and transporters were also differentially expressed (see the online Appendix at http://www.nephrogenomics.net/data/appendices/Hypertension2004).

Discussion

We chose to investigate the whole population of leukocytes and to pool samples from patients in a similar condition for this first assessment of gene expression in circulating leukocytes. As such, this does not allow conclusions as to whether a specific cell type could be more responsible for the observed changes. In this respect, it is of note that there was no difference in the total number of leukocytes and the contribution of the most frequently occurring cell types, neutrophils and lymphocytes, was similar in all groups. Phenotyping individual cell types, which will require isolation procedures that do not affect RNA composition, remains a challenge. Also, using the microarray technique to assess gene expression has limitations regarding its sensitivity and specificity. Despite these limitations, the study uncovers novel aspects of essential hypertension.

The first issue that was investigated was whether leukocytes of hypertensive patients differentially express oxidative stress- and inflammation-related genes. Indeed, expression of several pro-oxidant genes was increased, whereas antioxidant genes decreased. Most remarkable was the observed modulation in inflammation- and cytokine-related genes. In Figure 3, this information is summarized, focusing on chemokines and cytokines. Expression of several anti-inflammatory interleukin receptors was decreased. A shift to proinflammatory interleukins might have induced the selective expression of inhibitors of cytokine signaling. Recently, the JAK/STAT pathway and its intrinsic inhibitors have been the focus of several articles proposing that imbalance between these signaling elements can strongly modulate the impact of external proinflammatory and anti-inflammatory factors such as IL-6 and IFNγ. When this picture of alterations in gene expression is compared with that suspected to be operational in advanced atherosclerosis, the resemblance is striking.

In the patients who were treated, virtually none of the observed changes could be detected. In this respect, it should be noted that using the approach of pooled samples, it cannot be concluded that changes normalized to the same extent in each individual. Additional information on the observed changes in gene expression can be found in the online appendix (www.nephrogenomics.net/data/appendices/Hypertension2004).
The second issue that was addressed was whether leukocyte gene expression in hypertension displays differential regulation of genes associated with blood pressure control. Some of the expression changes such as increased EDN2 go along with suspected increased levels of endothelin in essential hypertension. However, an increase in ANP-A receptor and a decrease in ECE1 expression is more difficult to interpret in this light. ANP receptor expression has been described in monocytes and macrophages and could follow an adaptive response to volume expansion. It is tempting to speculate about the relation of the many differentially regulated channels and transporters to changes in vascular contractility and renal tubular function associated with hypertension; however, this requires further investigation of specific genes. Two observations potentially link blood pressure regulation and inflammation. First, 5-HT receptors were induced and have been implicated to play a role in several hypertension forms and animal models, in particular the HTR2B receptor. These receptors have also been reported to modulate T cell and B cell function. Second, AT1 receptor expression was increased. The AT1 receptor has been shown to mediate the activation of NADPH-oxidase by angiotensin II in several cell types, and thereby could induce an inflammatory response. It is of note that this study is a first step in the characterization of gene expression responses of leukocytes in the hypertensive state. To further substantiate the hypothesis that Ang II could be involved in the induction of an inflammatory response via leukocytes in essential hypertension, the increase in AT1 receptor gene expression needs followed-up at protein and functional levels. Such studies should also address whether the treatment acts via a decrease in blood pressure or via a decrease in systemic Ang II levels.

Taken together, for the first time, to our knowledge, we report large-scale gene expression changes in leukocytes of hypertensive patients and we identify characteristics of inflammation. Changes in genes coding for proteins that are involved in blood pressure regulation, in particular the AT1 receptor, suggest that leukocytes could form a link between blood pressure regulation and inflammation. Strikingly, expression profiles of patients that had regained normal blood pressure with treatment were not different from normotensive controls. This study opens the way for more detailed investigation of expression profiles of patients with cardiovascular risks and to tailor-made treatment for them.

Perspectives

Challenges ahead are matching the expression profile of the individual patient with his or her cardiovascular risk. In breast cancer, (semi)supervised learning strategies on expression profiles have resulted in a more accurate prognosis. Perhaps such an approach, in a larger sample, will enable identification of individuals not effectively responding to therapy in terms of cardiovascular risk despite effective blood pressure lowering. From the present study, it cannot be concluded that in each individual, expression profile is normalized. Further studies will be needed to determine whether normalization of a gene expression profile in an individual also leads to normalization of the cardiovascular risk. In the present study, ACE inhibition and β-blockade were applied as antihypertensive drugs. Both lead to inhibition of the renin-angiotensin system and of the sympathetic nervous system. It remains open as to which of these systems or the blood pressure per se was responsible for the alterations in gene expression in the untreated subjects with hypertension. As such, the strategy used in the present study could be further refined to be applied in individual patients.

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