Gene expression profile of endothelin-1-induced growth in glomerular mesangial cells

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Mishra, Rangnath, Patrick Leahy, and Michael S. Simonson. Gene expression profile of endothelin-1-induced growth in glomerular mesangial cells. Am J Physiol Cell Physiol 285: C1109–C1115, 2003. First published July 9, 2003; 10.1152/ajpcell.00105.2003.—Endothelin (ET)-1 is a vasoconstrictor and mitogen involved in vascular remodeling. Changes in gene expression that underlie control of cell growth by ET-1 remain poorly characterized. To identify pathways of growth control we used microarrays to analyze ET-1-regulated gene expression in human mesangial cells, an important ET-1 vascular target cell in vivo. Statistical assessment of differential expression (significance analysis of microarrays) revealed upregulated transcripts for growth factors [heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), fibroblast growth factor (FGF), interleukin (IL)-6] and downregulated transcripts for genes that inhibit growth (BAX, p27KIP1, DAD1). Consistent with the gene expression profile, quantitative RT-PCR and Western blotting confirmed induction of HB-EGF by ET-1. To test a functional role for HB-EGF in ET-1 signaling, we showed that exogenous HB-EGF stimulated phosphorylation of ErbB1 and growth of mesangial cells. ET-1-induced proliferation was blocked by an ErbB1 receptor-selective kinase inhibitor and by a specific ErbB1 receptor-neutralizing antibody. Proliferation in response to ET-1 was also inhibited by neutralizing antisera against human HB-EGF. Together, these results provide data for modeling ET-1 pathways for growth control and suggest a specific role for HB-EGF gene induction in mesangial cell growth in response to ET-1.

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the effects of ET-1 in vascular cells. We therefore used high-density oligonucleotide microarrays to profile changes in mRNA transcripts in human mesangial cells stimulated to proliferate with ET-1.

MATERIALS AND METHODS

Cell culture and experimental design. Mesangial cell strains (passages 4–7) were isolated from human kidney obtained at autopsy and characterized as previously reported (13, 28). Human kidney cortex was obtained from the National Cancer Institute Cooperative Human Tissue Network at Case Western Reserve University. All studies were approved by the Institutional Review Board of the University Hospitals of Cleveland. Cells were maintained in RPMI 1640 at Case Western Reserve University. All studies were preceded by factor VIII, keratin, or common leukocyte antigen. Semi-confluent cultures were made quiescent for 24 h in 0.5% FBS and then stimulated with 100 nM ET-1 (American Peptides, Sunnyvale, CA) for 0.5, 1, 6, and 24 h. Total RNA was isolated and mRNA transcripts were analyzed as described in Microarray analysis of RNA transcript expression. Each time course was conducted in three independent experiments and hybridized to individual microarray chips.

Microarray analysis of RNA transcript expression. Target RNA from mesangial cells was labeled and hybridized to high-density oligodeoxynucleotide microarrays from Affymetrix (Santa Clara, CA) as previously described (23). Biotin-labeled cRNA (15 μg) was hybridized to Human Genome U95A microarrays for 16 h at 45°C and after stringent washing in a microfluidics station, bound cRNA was stained with R-phycocerythrin streptavidin (Molecular Probes, Eugene, OR) and scanned before and after antibody amplification. Fluorescence intensities were analyzed with a laser confocal scanner (Hewlett-Packard). Image output files were inspected for hybridization artifacts, and on the basis of fluorescence intensity differences between perfect match and mismatch probes, absolute mRNA detection call (present, marginal, or absent) was determined with a P value calculated from a Wilcoxon signed-rank test (Microarray Suite 5.0, Affymetrix). Default parameters optimized by the manufacturer for this microarray chip were used for all detection and analysis. The U95A microarray represents 12,625 gene sequences derived from expressed sequence tag (EST) clusters in Build 95 of Unigene. Of these sequences 10,929 are annotated genes [either GenBank or The Institute for Genomic Research (TIGR)], 1,629 are unannotated ESTs, and the remainder are duplicate or control probe sets.

Analysis of RNA expression data. The transcript signal, which indicates mRNA transcript abundance, was used to determine whether transcript levels were altered in ET-1-stimulated cells at different time points. Scanned images were globally scaled to a target intensity of 1,500 to facilitate comparison of transcript levels from different time points. Significant changes in transcript level were determined by using the Wilcoxon signed-rank test to compare individual probe pair data for each transcript between the zero-time and ET-1-stimulated arrays. From the resultant P values, change calls (increase, decrease, no change) were assigned to each transcript at every time point. Each time course (n = 3) was analyzed as an independent experiment. As a first-pass analysis, for differential expression, a gene was identified if at a single time point it was assigned an increase or decrease call in two of three independent experiments, which resulted in 644 genes. This data set was further refined with significance analysis of microarrays (SAM) to identify changed genes that were highly reproducible (32). SAM assigns a score to each gene in a gene expression profile on the basis of a change in gene expression relative to the standard deviation of repeated measurements. A relative difference score, d(t), was then assigned relative to the standard deviation of the repeated measurements for each gene. This approach generated a list of 122 genes, with a false discovery rate of 13 genes. Hierarchical clustering using pairwise average linkage (10) was performed with Cluster and TreeView, developed by Eisen and coworkers (10), with the Pearson correlation coefficient as the metric of similarity. To identify functional classification of the ET-1-stimulated transcripts, the Gene Ontology (GO) database was used (2).

Validation of mRNA levels by real-time RT-PCR. Isolation of total RNA after ET-1 stimulation and synthesis of cDNA was carried out as described in Microarray analysis of RNA transcript expression. A different subset of genes was used for quantification of mRNAs by LightCycler RT-PCR (Roche Molecular Biochemicals) as previously described (23). We designed and synthesized primers for human heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) that spanned introns 1–5; upstream, GGTGCTGAAGCTTTCTGGCTG; downstream, ATTATGGGAGGCCCAATCTTAGAC. Probes for GAPDH mRNA were as previously described (23). Agarose gel electrophoresis of the RT-PCR products confirmed that only one band of the predicted molecular weight was present. To construct a standard curve, known concentrations of cDNA specific for HB-EGF and GAPDH were run in parallel to the experimental samples. A melting curve recorded at the end of the reaction was used for correction of the amplification curve. Amounts of mRNA in ET-1-stimulated samples were calculated from the standard curve plot of crossing point in the log-linear range vs. the log of copy number. Amounts of the mRNA transcripts are presented in reference to those in the untreated cells.

Western blotting of HB-EGF and phosphoErbB1 protein in ET-1-stimulated mesangial cells. Western blotting was performed as previously described with minor modifications (13). After addition of ET-1 or 5 ng/ml recombinant human HB-EGF (R&D Systems, Minneapolis, MN), cells in 60-mm dishes were washed once in Dulbecco’s phosphate-buffered saline and scraped into 0.2 ml of sample lysis buffer. The lysate was vortexed and boiled for 5 min, aliquots were resolved on 4–20% SDS-PAGE gradient gels, and proteins were transferred to 0.2-μm nitrocellulose filters. Transferred proteins were stained with Ponceau S to confirm equal protein loading, and the filter was blocked overnight at 4°C. Membranes were probed with the appropriate antibody as follows: 1:2,000 of an affinity-purified goat anti-human HB-EGF antiserum (R&D Systems); 1:1,000 of rabbit anti-P-Tyr 1068 ErbB1 (Cell Signaling, Beverley, MA), which recognizes the active form of ErbB1; and 1:5,000 of a mouse monoclonal antibody against β-actin (clone AC-74, Sigma, St. Louis, MO). After extensive washing, the appropriate peroxidase-labeled secondary antibody in blocking buffer was added and the blots were detected by chemiluminescence. Typical exposure times were 30–60 s.

Mesangial cell proliferation. Cells (5 × 10^5) were plated into 12-well plates and made quiescent in 0.5% FBS for 24 h before stimulation with 100 nM ET-1 or 5 ng/ml HB-EGF. At 24 and 48 h after stimulation, the number of cells in

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trypsinized monolayers was quantified with a hemocytometer. In some experiments, serum-starved cells were pretreated 1 h before addition of ET-1 with 500 nM AG-1478 (Calbiochem, La Jolla, CA) in 0.001% DMSO, a selective inhibitor of the EGF receptor (EGFR) kinase (20), with 10 μg/ml of a monoclonal neutralizing antibody (clone LA1, Upstate Biotechnology, Lake Placid, NY) against an extracellular epitope of EGFR (29), or with 10 μg/ml of a neutralizing goat antibody against human HB-EGF. Mesangial cells were pretreated with DMSO or with an equimolar concentration of mouse IgG1 or goat IgG (Sigma) to control for nonspecific effects. Each experiment was replicated three times in duplicate, and statistical significance was calculated with an unpaired t-test.

RESULTS

Identification of genes regulated by ET-1. Mesangial cells were serum starved for 24 h before addition of 100 nM ET-1 for 1, 6, and 24 h and measurement of relative mRNA levels. ET-1 stimulates mesangial cell proliferation under these conditions (23). SAM analysis identified 122 genes reproducibly regulated by ET-1, which are visually represented as a hierarchical cluster of genes and experiments (Fig. 1A). The utility of SAM to detect differentially regulated genes is supported by the fact that all replicates of individual time points clustered together (Fig. 1A). Distinct patterns of gene expression emerged from the cluster. Group 1 held genes strongly repressed by ET-1 and had many unannotated transcripts (Fig. 1A). Group 2 contained genes that were induced at 6 and/or 24 h. Many genes in group 2 have well-established roles in inflammation such as interleukin (IL)-6 and macrophage chemotactic protein-1 (MCP-1). The pattern of expression in group 3 was less obvious but contained mostly genes that were induced at 6 h and subsequently repressed at 24 h. Group 4 consisted of transiently induced genes including activating transcription factor-3 (ATF-3), fibroblast growth factor (FGF)-5, early growth response (EGR)-1 transcription factor, and HB-EGF. Individual expression profiles for BAX, HB-EGF, MCP-1, and IL-6 with means ± SD demonstrate the reproducibility of genes identified by SAM from the microarray data set (Fig. 1B). Genes represented by multiple probe sets on the array, such as BAX and MCP-1, were concordant (Fig. 1B). A complete list of genes in Fig. 1A is available at www.cwru.edu/med/simonson. Most of these genes have not previously been associated with ET-1 stimulation.

To identify candidate genes involved in ET-1 control of mesangial cell growth, we filtered the SAM data set

Fig. 1. Hierarchical cluster of genes regulated by endothelin (ET)-1 in mesangial cells. A: quiescent mesangial cells were stimulated by 100 nM ET-1 for 1, 6, and 24 h. Genes reproducibly induced (red) or repressed (green) by ET-1 were identified by significance analysis of microarrays (SAM) (n = 122), clustered by gene and time point (n = 3), and displayed as a hierarchical cluster of the log2 signal ratio. A position in the cluster for specific genes referred to in the text is indicated beside the cluster with a GenBank identifier. B: expression profiles extracted from the cluster for BAX, heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), macrophage chemotactic protein (MCP)-1, and interleukin (IL)-6 are means ± SD (n = 3) of the log2 signal value; BAX and MCP-1 were represented by 3 and 2 probe sets, respectively.
for transcripts with a gene ontology of “cell growth” (2). The gene in this set with the highest d(i) score was HB-EGF, and the significance of HB-EGF gene expression was analyzed as described in Gene and protein expression patterns for HB-EGF.

**Gene and protein expression patterns for HB-EGF.** We validated mRNA and protein expression for HB-EGF in response to ET-1. The HB-EGF locus encodes the heparin-binding EGF-like growth factor, and ET-1 increased HB-EGF mRNA measured by RT-PCR (Fig. 2A). Maximal fold induction of HB-EGF mRNA was higher (2.7-fold) when measured by RT-PCR. ET-1 also increased expression of a 25-kDa transmembrane HB-EGF protein beginning at 3 h and declining to basal levels at 24 h (Fig. 2B). Lesser amounts of 16- to 19-kDa sHB-EGF, which represent a fraction of the proteolytically processed and secreted form of HB-EGF that remains with the cell monolayer (12), were also detectable. Together, these results suggest that the SAM filter coupled with gene ontology correctly identified differential expression of a gene in the microarray data set that can be further tested for a functional role in growth control by ET-1 as shown in HB-EGF contributes to mesangial cell growth by ET-1.

**HB-EGF contributes to mesangial cell growth by ET-1.** We next asked whether induction of HB-EGF mRNA by ET-1, which has not to our knowledge been identified with other screening techniques, contributes to proliferation of mesangial cells treated with ET-1. Addition of recombinant human HB-EGF to serum-starved mesangial cells increased tyrosine 1068 phosphorylation of ErbB1/EGFR (Fig. 3A), which demonstrates that human mesangial cells express functional ErbB1. ErbB1 phosphotyrosine 1068 is the site for Grb2 SH2 domain binding, which results in Ras and MAPK activation through a Grb2/Sos-1 mechanism (27). In some cells ET-1 has been reported to rapidly transactivate ErbB1 (6), but addition of ET-1 to human mesangial cells for up to 1 h caused only minor ErbB1 phosphorylation at tyrosine 1068 that was difficult to detect reproducibly by Western blotting (data not shown). However, longer term incubation with ET-1 increased the level of phosphoErbB1 at 4–8 h (Fig. 3B), consistent with the delayed time course of HB-EGF induction by ET-1. Addition of recombinant HB-EGF increased proliferation of mesangial cells as measured by cell counts (Fig. 3C). HB-EGF nearly doubled the number of cells after 24 h. These results demonstrate that HB-EGF activates ErbB1 receptors in mesangial cells.

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**Fig. 2. ET-1 increases HB-EGF mRNA and protein levels.**

_A_: mRNA levels for HB-EGF analyzed independently by RT-PCR compared with results from the microarray (MA) (n = 3 independent experiments). _B_: Western blotting of HB-EGF protein showed upregulation of 25-kDa membrane-associated HB-EGF and a 19-kDa HB-EGF previously identified as sHB-EGF, a fraction of the proteolytically processed and secreted form of HB-EGF that remains with the cell monolayer (12). Similar results were observed in 2 independent experiments, and each blot was stripped and reprobed for β-actin.

**Fig. 3. Exogenous HB-EGF activates ErbB1 and is a mitogen for human mesangial cells.** _A_: quiescent cells were treated with 5 ng/ml recombinant human HB-EGF, and the activity of ErbB1 was assessed by Western blotting with an ErbB1 anti-phosphotyrosine 1068 antibody. Total ErbB1 was measured by stripping each blot and reprobing with nonactivation state-specific antibody. _B_: ET-1 at 100 nM was added for 1 to 24 h, and ErbB1 activation was measured by Western blotting as described in _A_. Similar results were observed in 3 independent experiments. _C_: recombinant human HB-EGF (5 ng/ml) was added to quiescent cells, and cell number was counted at 24 and 48 h. Data are means ± SE for 3 independent experiments in duplicate. pp, Phosphoprotein; p, protein.
cells and stimulates mitogenesis. Furthermore, the delayed activation of ErbB1 by ET-1 corresponded to the time course of HB-EGF induction in the microarray results and Western blots.

To investigate whether HB-EGF contributes to growth stimulated by ET-1, cells were pretreated with a selective ErbB1/EGFR antagonist, AG-1478 (20). ET-1 increased mesangial cell number 2.3-fold over control at 48 h, and AG-1478 prevented this increase (Fig. 4A). DMSO alone (vehicle control) had no effect. Quiescent mesangial cells were also pretreated with a neutralizing monoclonal antibody (clone LA1) that recognizes an epitope of the extracellular domain of ErbB1/EGFR to inhibit ligand activation of the receptor (29). MAb LA1 inhibited ET-1-induced cell growth, whereas a mouse IgG1 isotype control added at the same concentration had no effect (Fig. 4B). Collectively, these results support a model in which ET-1-induced proliferation requires ligand-activated ErbB1.

To test specifically whether induction of HB-EGF by ET-1 is involved, we treated mesangial cells with an affinity-purified anti-human HB-EGF antibody that neutralizes the biological activity of HB-EGF (1, 33). We confirmed that the antibody neutralized HB-EGF-induced proliferation in human mesangial cells (Fig. 4C). Addition of the neutralizing antibody prevented proliferation stimulated by ET-1 (Fig. 4C). Proliferation by ET-1 was not affected by an equivalent amount of goat IgG control antibody. These results are consistent with a specific role for HB-EGF in proliferation of mesangial cells in response to ET-1.

**DISCUSSION**

ET-1 is an endothelial cell-derived pressor peptide that controls expression of genes critical for adaptive remodeling of vascular cells in response to injury. Evidence from experimental and clinical studies suggests that ET-1 directs growth, fibrosis, and inflammation in a variety of vascular cells. In this study, we identify a set of genes differentially regulated by ET-1 in mesangial cells and demonstrate a specific role for HB-EGF gene induction in ET-1-stimulated proliferation.

The ET-1 gene expression profile in mesangial cells suggests a model for utilization of HB-EGF in mitogenic signaling. In specific cell types, G protein-coupled receptor agonists such as ET-1, thrombin, or carbachol...
rapidly transactivate ErbB1. Transactivation occurs only in certain cell types and proceeds by intracellular signals (7, 9, 21, 22) or by metalloproteinase-dependent cleavage and release of soluble HB-EGF (25). The mechanism we propose in mesangial cells is different. Previous models invoke heterologous modulation of ErbB1 that occurs too quickly to require induced synthesis of EGF-ligands (6). Our results suggest an additional, longer term pathway involving HB-EGF gene induction rather than short-term transactivation of ErbB1, which is difficult to detect in ET-1-treated mesangial cells. Transactivation of ErbB1 by G protein-coupled receptor agonists is apparently highly dependent on cell context and the activation state of protein kinase C (7, 21). In the present experiments, ET-1 increased HB-EGF mRNA measured by microarray and RT-PCR, and the increase in membrane-associated HB-EGF protein followed the gene expression profile. ET-1 increased HB-EGF protein maximally at 4–5 h, after which time HB-EGF slowly declined back to basal levels at 24 h. The delayed time course of ErbB1 activation by ET-1 in mesangial cells paralleled HB-EGF induction.

That HB-EGF gene expression contributes to ET-1-stimulated cell growth was suggested by three lines of evidence. A selective inhibitor of ErbB1 attenuated proliferation, as did a neutralizing antibody against ErbB1. A specific role for HB-EGF was suggested by the ability of an HB-EGF-neutralizing antibody to block proliferation in cells treated with ET-1. Induction of HB-EGF might play a more general role in mesangial cell growth; we previously demonstrated (23) that serum induces HB-EGF mRNA and protein in mesangial cells and that inhibition of ErbB1 attenuates cell growth. However, the present study supports a more specific role for HB-EGF as indicated by experiments with the HB-EGF-neutralizing antisera and the fact that genes for other ErbB1 ligands were not upregulated in the present study with ET-1. Previous experiments showed that HB-EGF stimulates proliferation of mesenchymal cells by both autocrine and juxtacrine mechanisms (12), but the present experiments do not address this issue. An important caveat is that in some cells HB-EGF can signal through ErbB4 (16, 26); the expression of ErbB4 has not been well characterized in human mesangial cells, so it is possible that ET-1-stimulated HB-EGF also contributes to proliferation by activating ErbB4.

In addition to its growth factor activity, several reports demonstrate a role for ET-1 in accumulation of the mesangial matrix in chronic renal injury (5, 11, 14, 30). In particular, ET-1 is an important mediator of collagen I accumulation in the glomerulus (5, 11, 11). An analysis of the ET-1 gene expression profile by GO functional classification provides additional insights into how ET-1 might promote matrix accumulation in the mesangium (Fig. 5). Significant increases were observed in three matrix protein genes: collagen type 1 \(\alpha_1\) and \(\alpha_2\) and a chondroitin sulfate proteoglycan, versican. Induction of thrombospondin 2 mRNA by ET-1 would likely enhance collagen fibrillogenesis and might modulate the cell surface properties of mesangial cells to affect cell adhesion and migration (19). ET-1 increased transcripts for two cytokines that increase mesangial matrix production, transforming growth factor (TGF)-\(\beta\) and IL-6. In addition to these matrix-promoting genes, ET-1 also stimulated transcripts for two genes that inhibit fibrinolysis and matrix protein degradation: tissue inhibitor of matrix metalloproteinases (TIMP)-3 and plasminogen activator inhibitor (PAI)-2 (Fig. 5). TIMP-3 is a natural inhibitor of the matrix metalloproteinases that degrade extracellular matrix, and PAI-1 plays a pivotal role in progressive renal disease in both glomerulosclerosis and tubulointerstitial fibrosis (8). Thus ET-1 regulates expression of several genes whose coordinated expression might promote matrix expansion and accumulation in the mesangium in vivo.

In conclusion, we have constructed a gene expression profile for analyzing regulation of cell growth by ET-1. In addition, a specific role for HB-EGF gene induction, as predicted by the microarray results, was demonstrated in the proliferative response to mesangial cells to endogenous ET-1.

DISCLOSURES

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