Short Technical Report

Microarray Gene Expression Profiling of Angiogenesis Inhibitors Using the Rat Aortic Ring Assay

BioTechniques 33:664-670 (September 2002)

T.G. Zogakis, N.G. Costouros, E.A. Kruger, S. Forbes, M. He, M. Qian, A.L. Feldman, W.D. Figg, H.R. Alexander, E.T. Liu, E.C. Kohn, and S.K. Libutti

National Cancer Institute,
Bethesda, MD, USA

ABSTRACT

The rat aortic ring assay has been previously described as a useful ex vivo model for analyzing the biological activity of various inhibitors of angiogenesis. Rat aortic rings are exposed to antiangiogenic agents for a five-day incubation period. Then, the degree of microvessel outgrowth from the rings is analyzed and quantified. In contrast to most in vitro angiogenesis assays, the rat aortic ring model provides a unique microenvironment to evaluate the interaction of various cell types and biological factors for their influence on angiogenesis. Microarray analysis is an accepted method for the evaluation of gene expression profiles and can be used to better understand changes in gene expression that occur when rat aortic rings are exposed to a particular biological agent. Here we describe a method of using microarray technology to evaluate the modulation of gene expression in angiogenesis using the rat aortic ring assay.

INTRODUCTION

The process of angiogenesis involves interactions between multiple cell types and is therefore difficult to mimic using in vitro endothelial cell culture. Thus, the analysis of most in vitro models of angiogenesis using microarray technology may miss important changes in gene expression due to the influences of non-endothelial cells. Conversely, in vivo models often contain a small fraction of endothelial cells; thus, changes in endothelial cell gene expression are masked by non-endothelial cells.

Microarray technology has provided an invaluable tool for the analysis of gene expression data (2). We have adapted a previously described ex vivo rat aortic ring model for microarray analysis that includes an enriched endothelial cell population as well as various cell types and biological factors important for the study of angiogenesis (6). Thus, microarray analysis of the rat aortic ring model is a valuable tool for the discovery of genes and pathways involved in angiogenesis and can help clarify the mechanism of an anti-angiogenic agent at a molecular level.

MATERIALS AND METHODS

Ring Assay

Each well of a 12-well tissue culture-grade plate was covered with 250 μ L Matrigel™ (BD Biosciences, San Jose, CA, USA) and allowed to gel at 37°C for 30 min. For rat aortic rings, the thoracic aorta was harvested from 8- to 10-week-old male Sprague-Dawley rats,

taking care to remove any surrounding fibroadipose tissue. Animals were handled as per National Institutes of Health (NIH) (Bethesda, MD, USA) approved guidelines and were treated by the Institutional Animal Care and Use Committee (IACUC) (Memphis, TN, USA) approved protocols. Human saphenous veins were acquired during another procedure requiring the harvesting of the human saphenous vein. All procedures were performed by National Cancer Institute (NCI) (Gaithersburg, MD, USA) IRB-approved protocols. Vessels were then sectioned into 1-mm-long lengths and rinsed eight times with endothelial cell growth media (EGM-2) (BioWhittaker, Walkersville, MD, USA). Each vessel section was placed into a separate Matrigel-coated well. The wells were then covered with an additional 250 μ L Matrigel each and again allowed to gel for 30 min. The rings were incubated for 24 h at 37°C, 5% CO₂ in 2 mL EGM-2. Afterwards, the media were removed and replaced with 1 mL endothelial cell basal medium (BioWhittaker) supplemented with 2% FCS and 10 μ g/mL gentamicin with or without the drug. The drug carboxyamidotriazole (CAI) was applied at a concentration of 12 μ g/mL. After five days of incubation, microvessel outgrowth from rings was quantified using Image 1.62 Software (NIH). A digital image was created upon photographing each ring. Pixel density was then computed, and the mean pixel area from duplicate rings was calculated.

RNA Extraction and Amplification

RNA extraction. Rings with their associated vessel outgrowths and part

of the surrounding Matrigel were removed *en bloc* using a punch biopsy and spatulae. This ensured that the outgrowths with potential cells of interest were indeed removed and not left behind. The rings were then snap-frozen in liquid nitrogen. TRIzol® (Invitrogen, Carlsbad, CA, USA) RNA extraction was performed, yielding nanogram quantities of total RNA.

cDNA synthesis. Total RNA was primed with 2 µg synthetic oligonucleotide (oligo-dT) containing the phage T7 RNA polymerase promoter. The RNA/primer mixture was subjected to a 10-min heat denaturation at 70°C. First-strand synthesis was performed with SUPERSCRIPT II™ reverse transcriptase (Invitrogen). Second-strand cDNA synthesis was then performed with *E. coli* DNA polymerase I and RNase H (both from Invitrogen) for 2 h at 16°C. cDNA was made blunt-ended by incubation with 20 U T4 DNA polymerase (Invitrogen) for 5 min at 16°C. cDNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1) and then purified.

Antisense RNA synthesis. T7 Megascript™ kit (Ambion, Austin, TX, USA) was used per the manufacturer's instructions. The reaction mixture was incubated at 37°C for 4 h. RNA was then extracted by phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of 95% ethanol at -20°C.

Second-round amplification. Amplified RNA (aRNA) was primed with 1 µg random hexamer primers (Invitrogen) and subjected to a 10-min heat denaturation at 70°C. First-strand cDNA synthesis was performed as previously described. Oligo-dT primer (0.5 µg) was then added, and the first-strand cDNA/primer mixture was heat-denatured at 70°C for 5 min. Second-strand cDNA synthesis and in vitro transcription were performed as described above. RNA was then purified using RNeasy® mini kit (Qiagen, Valencia, CA, USA) per the manufacturer's instructions.

Two rounds of amplification using a modified Eberwine method (8), followed by RNeasy purification, yielded 30–60 µg mRNA from 4–6 rings. The RNA A₂₆₀:A₂₈₀ ratio was 2.6–2.9, suggesting high purity. Formaldehyde gel

analysis (1.5% agarose and 0.6 M formaldehyde) of the aRNA confirmed purity and quantity. This method was similar to the Eberwine method previously described (2,9).

Microarray Analysis

Target labeling by reverse transcription. Five micrograms of amplified mRNA were primed with 6 µg random hexamer primer (Invitrogen) and subjected to a 5-min heat denaturation at 65°C. Labeled cDNA target was generated using 4 mM Cy3- or Cy5-dUTP (Amersham Biosciences, Piscataway, NJ, USA) and SUPERSCRIPT II incubated with primed RNA for 60 min at 42°C. RNA was hydrolyzed at 65°C with 1 M NaOH. Cy5- and Cy3-labeled targets were combined appropriately and then purified and concentrated using Microcon® YM-30 (Amicon, Bedford, MA, USA) and Tris-EDTA (pH

7.4) washes. Then, 10 µg COT-1 human DNA (Invitrogen), 4 µg yeast RNA (Sigma, St. Louis, MO, USA), and 10 µg poly(A) (Amersham Biosciences) were added to the purified target.

Prehybridization. Each microarray chip (6.5K rat or human, respectively) (Advanced Technology Center, National Cancer Institute) was prehybridized at 42°C for 1 h with prehybridization buffer (5× SSC, 0.1% SDS, and 1% BSA). Slides were then washed with distilled water and then isopropanol and allowed to air-dry.

Hybridization. A 2× hybridization buffer consisting of 50% formamide, 10× SSC, and 0.2% SDS was added in equal part to each target after denaturing the target at 100°C for 1 min. Targets were then incubated to microarrays in a humidified, water-tight chamber at 42°C for 16 h.

Hybridization washes. After hybri-

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

dization, slides are washed with 2× SSC and 0.1% SDS and then gradually decreasing concentrations of SSC (1×, 0.2×, and 0.05×). They are then spun dry.

Arrays were scanned using a GenePix® 4000 Scanner (Axon Instruments, Foster City, CA, USA), and the data set was analyzed and extracted using GenePix Pro 3.0 Microarray Analysis Software (Axon Instruments). The mAdb tools (Center for Information Technology, NCI) were used to perform microarray normalization and statistical analysis. A 1.5- and 3-fold difference in expression corresponds approximately to 66% and 99% confidence of expression change on a single array. Multiple arrays were performed to minimize the number of false-positive genes due to multiple sample testing.

Real-Time Quantitative PCR

Real-time quantitative PCR analysis was carried out as described previously (5), with minor modifications. Briefly, measurements were performed using the GeneAmp® 5700 Sequence Detector (Applied Biosystems, Foster City, CA, USA). Primers and probes (BioServe Biotechnologies, Laurel, MD, USA) were designed using PrimerExpress™ software (Applied Biosystems), except for rodent GAPDH, which was available commercially (Applied Biosystems). Probes were 5'-labeled with FAM and 3'-labeled with TAMRA. Sequences were CDK110 (UniGene Cluster: Rn.25727): 5'-GCTTCTTCGCATTC-CACTTCAT-3', 5'-CGTGGAGGAA-AAGAAGATGTACAA-3', 5'-FAM-CCATTCATTATCGCCGCCCTTG-TA-MRA-3'; and PDGFRα (UniGene Cluster: Rn.55127): 5'-CGAGAAAGGCTT-CGTCCAGAT-3', 5'-CTGACCTGAT-GCAGGTTTACA-3', 5'-FAMAGGCC-CACCTTTGGCCATCTGG-3'. cDNA was generated from RNA samples (2 ng/assay) using MultiScribe® reverse transcriptase and random hexamers for priming (Applied Biosystems). Reaction conditions were 10 min at 25°C, 30 min at 48°C, and 5 min at 95°C. cDNA standards for each gene were generated by primer-specific amplification from rat cDNA. Copies were calculated using the molecular weight of each PCR product. Real-time PCR for samples and standards were conducted in a volume of 25

μL using AmpliTaq Gold® DNA polymerase (Applied Biosystems), sense and antisense primer concentrations of 900 nM each, and a target concentration of

250 nM (except for GAPDH, for which primer and target concentrations were 100 nM, per manufacturer's recommendations). Thermal cycling parameters

Figure 1. Rat aortic ring assay. Rat aortic rings treated with media alone (A) and CAI (B). Mean pixel density area (C) showing inhibition of microvessel outgrowth with CAI compared to rat aortic rings treated with media alone.

Figure 2. Microarray analysis. Hierarchical clustering (A) of 434 genes using non-centered metric Pearson correlation filtered by the criteria: Ratio ≤ or ≥ 1.5 in 4 of 4 arrays, Signal:Background > 2.0, and Spot Size > 25. Forty differentially expressed genes (B) were identified at a ratio of ≤ or ≥ 3.0 in 4 of 4 arrays with 26 genes up-regulated after treatment with CAI.

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

---

were 2 min at 50°C, 10 min at 95°C, 40 cycles each consisting of a 15-s denaturation step at 95°C and a 1-min annealing/extension step at 60°C. Standard curves were generated for each gene, and copy numbers were extrapolated for each sample. All assays were performed in duplicate and reported as the mean. The relative expression of each gene was normalized to that of GAPDH, and the expression ratio for each gene for CAI:media was calculated.

RESULTS

To illustrate this technique, a comparison was made between rat aortic rings incubated with either EGM-2 or CAI at 12 µg/mL. CAI has known anti-neoplastic and antiangiogenic effects in vivo and in vitro (3,4,7,9). It has been shown to inhibit consistently microvessel outgrowth in the rat aortic ring as-

say (1) and therefore serves as a useful substance to evaluate treatment related changes in the expression of genes involved in angiogenesis in this assay. After five days of incubation at 37°C, inhibition of microvessel outgrowth was observed with CAI-treated rings compared to control. Computer analysis of pixel density was performed, showing a significant degree of inhibition with CAI treatment (Figure 1).

To evaluate changes in gene expression, it was necessary to harvest the aortic rings after treatment for RNA isolation and amplification. To ensure that the outgrowths with potential cells of interest were indeed removed and not left behind, associated outgrowths and part of the surrounding Matrigel were removed using a punch biopsy and spatulae. Six to eight rings from each experimental group were combined to minimize biological noise. Microarray analysis using Cy5- and Cy3-

labeled, dUTP-generated targets (i.e., the mRNA populations of interest) were then performed, comparing RNA isolated from control treated rings to RNA isolated from CAI-treated rings. Thus, Cy3-labeled control RNA was combined with Cy5-labeled CAI RNA for forward analysis, and Cy3-labeled CAI RNA combined with Cy5-labeled control RNA was used for reciprocal analysis. Duplicate forward and reciprocal hybridizations were performed to ensure that genes were truly differentially expressed and not a result of labeling bias and systemic error. Reciprocity of data using a Cy5:Cy3 ratio of greater than 1.50 and a Cy3:Cy5 ratio of less than 0.67 on all four arrays was high, indicating that genes regulated by CAI on forward analysis were likewise influenced on reciprocal analysis at this intensity cut-off (Figure 2A). This indicates that extracted and amplified mRNA from the rat aortic ring model is

DRUG DISCOVERY

AND GENOMIC TECHNOLOGIES

adequate and of sufficient quality for microarray analysis.

Initially, genes that were 2-fold up- or down-regulated on all four arrays (two forward and two reciprocal) were defined as being differentially expressed. This yielded a list of 225 genes. More stringent criteria with a cut-off of 3-fold up- or down-regulated were then used, resulting in the identification of 40 genes, of which 26 were up-regulated by CAI treatment (Figure 2B). Given this list, further analyses of these genes can be performed to determine the magnitude of changes in gene expression and the relationships of various gene products. We have also completed microarray analysis of rat aortic rings treated with TNP-470, another known inhibitor of angiogenesis (10), to verify the use of this technology when using a different agent (data not shown).

To confirm the validity of expression ratios found on microarray analysis, we evaluated two genes, one with increased and one with decreased relative expression, using real-time quantitative PCR. Relative expression ratios determined using real-time quantitative PCR were concordant with expression levels observed in the corresponding microarray data (Figure 3).

Based on the rat aortic ring assay, a human saphenous vein ring model has also been developed (6), thus adapting the assay to human tissue. Recently, we have successfully applied these microarray methods to a human saphenous vein ring model. Microarray

analysis using a 6.5K human cDNA microarray showed 32 genes differentially expressed between CAI-treated human saphenous vein rings and media-treated human saphenous vein rings at a ratio of 3-fold up- or down-regulated. This demonstrates that microarray analysis can be successfully performed to identify differences in gene expression between human saphenous vein rings treated with different agents. Thus, the human saphenous vein ring system allows for the study of gene expression profile changes in human tissue.

DISCUSSION

Microarray analysis can be used to evaluate the regulation in gene expression that occurs within the cellular environment of the rat aortic ring or human saphenous vein ring assay. It serves as a useful tool to identify novel genes whose expression is influenced by the tested substance. Because the ring assays are *ex vivo* models, they provide a unique microenvironment where interactions of various cellular types and molecules can be studied. In contrast to many angiogenesis assays, this assay provides a microenvironment to evaluate the interaction of various cell types and biological factors for their influence on angiogenesis. Furthermore, by using the human saphenous vein ring system, cross-species differences are eliminated, and a more relevant model for developing human anti-angiogenic cancer therapies is available. In conclusion, microarray analysis of ring models of angiogenesis can help elucidate genes and pathways involved in angiogenesis and provide insight into how an agent modulates angiogenesis at a molecular level.

ACKNOWLEDGMENT

T.G.Z. and N.G.C. contributed equally to this work.

REFERENCES

1. Bauer, K.S., K.J. Cude, S.C. Dixon, E.A. Kruger, and W.D. Figg. 2000. Carboxyamido-triazole inhibits angiogenesis by blocking

the calcium-mediated nitric-oxide synthase-vascular endothelial growth factor pathway. *J. Pharmacol. Exp. Ther.* 292:31-37.

2. Eisen, M.B. and P.O. Brown. 1999. DNA arrays for analysis of gene expression. *Methods Enzymol.* 303:179-205.
3. Ge, S., S. Rempel, G. Divine, and T. Mikkelsen. 2000. Carboxyamido-triazole induces apoptosis in bovine aortic endothelial and human glioma cells. *Clin. Cancer Res.* 6:1248-1254.
4. Jacobs, W., T. Mikkelsen, R. Smit, K. Nelson, M. Rosenblum, and E. Kohn. 1997. Inhibitory effects of CAI in glioblastoma growth and invasion. *J. Neurooncol.* 32:93-101.
5. Kammula, U.S., K.-H. Lee, A.I. Riker, E. Wang, G.A. Ohnmacht, S.A. Rosenberg, and F.M. Marincola. 1999. Functional analysis of antigen-specific T lymphocytes by serial measurement of gene expression in peripheral blood mononuclear cells and tumor specimens. *J. Immunol.* 163:6867-6875.
6. Kruger, E., P.H. Duray, M.G. Tsokos, D.J. Venzon, S.K. Libutti, S.C. Dixon, M.A. Rudek, J. Pluda, et al. 2000. Endostatin inhibits microvessel formation in the *ex vivo* rat aortic ring angiogenesis assay. *Biochem. Biophys. Res. Commun.* 268:183-191.
7. Lambert, P., K. Somers, E. Kohn, and R. Perry. 1997. Antiproliferative and antiinvasive effects of carboxyamido-triazole on breast cancer cell lines. *Surgery* 122:372-379.
8. Van Gelder, R.N., M.E. von Zastrow, A. Yool, W.C. Dement, J.D. Barchas, and J.H. Eberwine. 1990. Amplified RNA synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. USA* 87:1663-1667.
9. Wasilenko, W.J., A.J. Palad, K.D. Somers, P.F. Blackmore, E.C. Kohn, J.S. Rhim, G.L. Wright, Jr., and P.F. Schellhammer. 1996. Effects of the calcium influx inhibitor carboxyamido-triazole on the proliferation and invasiveness of human prostate tumor cell lines. *Int. J. Cancer* 68:259-624.
10. Yanase, T., M. Tamura, K. Fujita, S. Kodama, and K. Tanaka. 1993. Inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and metastasis of human cell lines *in vitro* and *in vivo*. *Cancer Res.* 53:2566-2570.

Received 20 March 2002; accepted 8 May 2002.

Address correspondence to:

Dr. Steven K. Libutti
Surgery Branch
Building 10, Room 2B17
10 Center Drive
Bethesda, MD 20892, USA
e-mail: steven_libutti@nih.gov

For reprints of this or
any other article, contact
Reprints@BioTechniques.com

Figure 3. Validation of microarray expression levels using real-time quantitative PCR. Platelet-derived growth factor receptor α (PDGFR α , striped bars); cytochrome B apoprotein (CDK110, solid bars).