Gene expression profile in mouse myocardium after ischemia

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Lyn, Deborah, Xiaowei Liu, Nicole A. Bennett, and Nerimiah L. Emmett. Gene expression profile in mouse myocardium after ischemia. Physiol Genomics 2: 93–100, 2000.—This study was designed to elaborate a molecular profile of expressed genes during ischemic injury to the mouse heart after surgical constriction of the left coronary artery without reperfusion. A mouse cDNA array containing 588 known genes was used to compare gene expression in heart RNA after 24-h ischemia with control tissue. Alterations in gene expression on the array were supported by relative reverse transcription-polymerase chain reaction analysis after timed periods of ischemia. Decreased levels of the cell cycle regulator p18ink4 and the oxidative responsive gene glutathione S-transferase were accompanied by an upregulation of the genes associated with cardiac muscle development, α-myosin heavy chain and fetal myosin alkali light chain. Other stress responses elicited by cardiac injury included an induction of Egr-1 and Egr-3 transcription factors, as well as the apoptotic regulator Bax. Altogether, these findings indicate that expression of genes associated with a fetal transcription program may be involved with the post ischemic remodeling process in heart ventricles.

complementary deoxyribonucleic acid array; coronary artery occlusion; myosin; p18ink4; transcription factors

ISCHEMIC HEART DISEASE is a leading cause of mortality worldwide. The cellular and biochemical pathology associated with cardiac dysfunction has been extensively characterized in different animal models (6, 7, 12). However, the molecular mechanisms associated with ischemic injury are not well defined. Traditional approaches for studying gene expression are limited to analysis of a small number of genes in a defined physiological condition. Most gene expression studies are directed at a specific cellular pathway or stress response. Various animal and cell-based models have been used to study gene expression associated with cardiac ischemia that have included the renin-angiotensin system, apoptosis, ion channels, transcription factors, heat shock proteins, and antioxidant enzymes, among others (for review, see Refs. 2, 5, 8, 11, 23). The recent availability of cDNA expression arrays (27) provides an attractive strategy for elaborating an unbiased molecular profile of large number of genes during ischemic injury. This experimental approach offers the potential to identify molecules or cellular pathways not previously associated with ischemia.

Microsurgical techniques were employed to advance a mouse survival model of ischemia by adapting described procedures (15, 18) used to study coronary artery occlusion. Myocardial ischemia was produced by complete surgical occlusion of the left anterior descending (LAD) branch of the coronary artery without reperfusion. This approach avoids the difficulty of interpreting molecular insults due to ischemia vs. additional effects induced by reperfusion injury. Moreover, the rapid development of transgenic mice to study cardiovascular diseases (4, 13, 20) provides an impetus for the use of the mouse as an appropriate animal model of ischemia.

In this study, a cDNA mouse expression array containing 588 genes representing diverse biological functions was used to simultaneously compare changes in gene expression after 24 h ischemia with heart tissue from sham-operated mice. The cDNA array experimental approach provided a global profile of gene expression changes in heart ventricle tissue after coronary artery occlusion. The resultant hybridization pattern was confirmed by relative RT-PCR assays after defined periods of ischemia ranging from 0.5 h to 7 days. Alterations in transcription included genes associated with cardiac muscle development such as the cell cycle regulator p18ink4 and the structural proteins, α-myosin heavy chain (α-MHC) and fetal myosin alkali light chain (MLC). Expression of Egr-1 and Egr-3 (early growth response factor) were induced by ischemia, whereas a reduction in glutathione S-transferase may be indicative of disturbances caused by oxygen deprivation. Other stress responses to ischemia included an induction in expression of the apoptosis regulator Bax, which may contribute to cell death rather than alterations in transcription of the anti-apoptotic molecules. These responses in gene expression may represent a balance between the cardioprotective and degenerative processes that accompany myocardial ischemia.

METHODS

Surgical procedures. A mouse survival model of ischemia was adapted from described procedures (18). Adult male ICR mice (Harlan Sprague Dawley, Indianapolis, IN) at 21–25 g were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) by intraperitoneal injection. An endotracheal tube of polyethylene (PE) tubing size 90 was inserted through the mouth to maintain ventilation. A midline skin incision on the chest was made, and the muscle under the
incision was coagulated to prevent bleeding. Artificial respiration was achieved by connecting the tracheal tubing to a rodent ventilator (rate of 90 breaths/min) in which 100% oxygen was provided to the inflow of the ventilator (Harvard Apparatus, Holliston, MA). The chest was opened along the sternum, retracted by suture to expose the heart, and the pericardium was removed under a Zeiss dissecting microscope (Thornwood, NY). At the tip of the left auricle, the LAD branch of the coronary artery was visible, and complete surgical ligation was achieved with an 8-0 suture. A segment of PE-10 tubing was placed within the knot to prevent injury to the blood vessel. A pale area of muscle wall was noticed on the distribution area of the LAD branch, and the chest was closed with a 5-0 suture. The ventilator was not removed until the mouse became conscious. At stated time intervals of ischemia when the heart was collected, the knot was examined by injecting air into the LAD to confirm coronary occlusion. Sham-operated control mice underwent a similar surgical procedure but without ligation of the left coronary artery. The Animal Research Committee of Morehouse School of Medicine approved all animal procedures in accordance with NIH guidelines.

Measurement of left ventricle pressure. The right carotid artery was cannulated with PE-10 tubing for collecting hemodynamic data. After measurement of arterial blood pressure, the tubing was advanced into the left ventricle for monitoring ventricle pressure. The cannula was connected to a pressure transducer and analyzed using a heat performance analyzer (model HPA-410a; MicroMed, Louisville, KY). Mean ventricular pressure was calculated using the 200/1 integration software. Animals were maintained at 37°C by a heating lamp.

RNA isolation. Total RNA was prepared from the removed heart ventricle (left and right regions) using the monophasonic solution RNApure reagent (GenHunter, Nashville, TN) according to recommended procedures. Tissues from two to three animals were pooled to reduce animal variability differences and homogenized in RNApure reagent. Genomic DNA was removed by treatment with RNase-free DNase I for 45 min at 37°C followed by phenol/chloroform extraction and ethanol precipitation. Absence of genomic DNA contamination was determined by PCR amplification of actin gene. RNA integrity was verified on a MOPS-formaldehyde agarose gel prior to cDNA synthesis.

Synthesis of cDNA and array hybridization. The cDNA array hybridization technique relies on the principle of reverse Northern blotting. Known genes (cDNA fragments 200–500 bp in length) are immobilized in duplicate on a nylon membrane that is hybridized to a labeled cDNA probe representing expressed genes in a particular tissue. A mouse cDNA Expression Array was obtained from Clontech Laboratories (Palo Alto, CA), in which all procedures were followed as recommended with the following modifications. First-strand cDNA was synthesized from 15 µg total RNA by Moloney murine leukemia virus reverse transcriptase and 2 µl CDS primer (Clontech). Each cDNA reaction was conducted in a 20-µl volume containing 45 µCi [α-32P]dCTP. The labeled DNA probe was applied to each membrane at 0.5 × 10^6 cpm/ml and the hybridization proceeded overnight at 68°C. After stringent washing, membranes were exposed to Kodak Biomax MR X-ray film at −70°C for different time periods. The hybridization procedure represents a single array experiment, and significant changes in gene expression were confirmed by RT-PCR (see below).

Reverse transcription-polymerase chain reaction analysis. Five micrograms of total RNA was preheated for 2 min at 70°C with 250 ng of random primer. Reverse transcription was conducted at 42°C for 15 min followed by 48°C for 45 min in a 30-µl volume using Superscript II reverse transcriptase (Life Technologies, Grand Island, NY). The reaction mixture was heated at 75°C for 15 min to terminate the cDNA synthesis. For subsequent PCR assays, the cDNA mixture was diluted 1:5 with sterile water, and a 2-µl aliquot was used for each gene-specific amplification. Oligonucleotide primers for individual genes were obtained from Clontech and represent the same sequences used to amplify the cDNAs on the array. Gene-specific oligonucleotide primers were designed to mouse Egr-3 mRNA (21) that encompassed the region 438–460 (forward) and 629–604 (reverse). Amplification reactions were conducted in a 25-µl volume with an initial step of 94°C for 2 min. This was followed by 20–32 cycles of amplification depending on gene abundance in heart tissue. Each cycle was 20 s at 95°C, 30 s at 62°C, and 30 s at 72°C, with a final extension of 7 min at 72°C in the presence of 0.4 µM of each primer and 1.25 U Taq DNA polymerase (Fischer Scientific, Pittsburgh, PA). A two-step PCR cycle was used to amplify Egr-1 and Egr-3 genes, which consisted of 30 s at 95°C and 2 min at 68°C. The 18S rRNA gene was used as a reference control (Ambion, Austin, TX), as amplification of this gene was most likely to reflect total RNA steady-state levels and avoid the use of invariant housekeeping controls.

To ensure that the analysis was conducted at the linear range of the amplification assay, a range of cycle numbers were initially performed, and quantification conducted at a cycle number within the linear range. Independent reverse transcription reactions were conducted from two different RNA preparations (two ventricles each). Analysis of each gene-specific PCR assay represents the average of two amplifications from each reverse transcription reaction (four separate amplifications except where noted). Densitometric analyses of PCR amplifications were conducted on a Nucleodivision system from Nucleotech (San Mateo, CA).

Analysis of array data. A complete list of genes on the array can be accessed at the web site http://atlasinfo.clontech.com. The AtlasImage 1.0 (Clontech) software was used to compare gene expression profiles between sham and ischemia heart RNA. Signal intensities between the compared arrays were normalized using the Global Mode (to develop a normalization coefficient), which uses an average value based on all the expressed genes. Analysis was conducted after 12 h and 3 days of film exposure to ensure that hybridization intensity
was in the linear range and to collect data on low abundantly expressed genes.

RESULTS

A panoramic profile of genes associated with myocardial ischemia was determined using a mouse model of coronary artery occlusion adapted from established procedures that demonstrate tissue damage of the left ventricle (15, 18, 20). Total animal survival from surgical procedures was at least 95%. Measurement of mean left ventricle pressure (Fig. 1) was used to assess cardiac function after ligation of LAD branch of the coronary artery. A significant decrease in ventricle pressure was observed at all time points of ischemia, indicating loss of contractile function. Immediately after LAD ligation (30 s), left ventricle pressure declined more than twofold, with the lowest pressure observed after 24-h of ischemia.

A mouse cDNA array was used to examine the abundance of expressed genes in heart RNA after 24-h ischemia relative to RNA obtained from sham control hearts (Fig. 2). The array was divided into six different classes that encompassed the following biological groups: 1) oncogenes, tumor suppressors, and cell cycle regulators; 2) stress response, ion channels, and intracellular signal transduction modulators and effectors; 3) apoptosis, DNA synthesis, repair, and recombination; 4) transcription factors and DNA binding proteins; 5) receptors, cell-surface antigens, and cell adhesion; 6) cell-cell communication, cytoskeleton, and protein turnover. In addition, other cDNAs are present for normalizing mRNA abundance along with plasmid and bacteriophage DNAs as negative controls to confirm hybridization specificity.

Detectable hybridization levels were identified in 78 of the 588 (~13%) represented genes on the array in which a threshold intensity at least twice the background value was scored as a genuine signal (after a 3-day film exposure). Only nine genes produced a hybridization signal on the array membrane after a 12-h film exposure. A ratio of adjusted intensities was determined for all detected transcripts of which only six genes (listed in Table 1) showed a significant change (ratio value greater than 2.0) in expression levels after ischemia.
Table 1. Genes with altered gene expression after ischemia

<table>
<thead>
<tr>
<th>Functional Group (protein/gene)</th>
<th>Adjusted Intensity</th>
<th>Sham</th>
<th>Ischemia</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle regulator</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p18ink4; cdk4 and cdk6 inhibitor</td>
<td>3,382</td>
<td>1,346</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Stress response</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione S-transferase (microsomal)</td>
<td>5,582</td>
<td>1,935</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Transcription factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egr-1 2-Zn-finger regulatory protein</td>
<td>0</td>
<td>5,843</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Lung kruppel-like factor (LKLF)</td>
<td>2,954</td>
<td>5,978</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>Protein turnover/cytoskeleton</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal myosin alcali light chain</td>
<td>7,462</td>
<td>17,702</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Serine protease inhibitor hormol 6</td>
<td>1,512</td>
<td>3,439</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>α-C Cardiac myosin heavy chain</td>
<td>54,728</td>
<td>85,872</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Housekeeping</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>75,040</td>
<td>86,002</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>35,846</td>
<td>31,766</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>9,758</td>
<td>20,581</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>Ribosomal protein S29</td>
<td>24,694</td>
<td>24,214</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

"Adjusted intensity" was determined by subtracting the background value for each gene, multiplied by the normalization coefficient. Analysis presented was done on X-ray film after 3 days of exposure except for α-cardiac myosin heavy chain and housekeeping genes, in which intensity values were after a 12-h film exposure (see METHODS). Ratios are presented as either upregulated (↑) or downregulated (↓), except for genes listed under "Housekeeping," where ratios represent adjusted intensities of ischemia divided by sham. *Ratio is undefined as adjusted intensity is at background level for sham membrane. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Gene expression. Genes expected to produce steady-state mRNA levels such as ubiquitin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein S29 had ratio values of ~1.0 (Table 1), indicating that an equivalent specific activity of labeled cDNA was hybridized to each membrane. Genes on the cDNA array with a significant change in expression were subjected to further examination by semiquantitative RT-PCR analysis at timed intervals of ischemia (0.5 h to 7 days).

The most noticeable alteration in gene expression after 24-h ischemia was the induction of the transcription factor Egr-1, early growth response factor-1 (Fig. 2). Egr-1 (also known as nerve growth factor induced-A, krox-24, ZfF268, and T158) is a prominent member of the Egr gene family that was initially discovered as a factor induced by serum stimulation in nondividing cells (29). Other mitogenic inducible transcription factors on the array such as c-fos, c-jun (undetected), and jun-D appeared unaffected by ischemia. Alterations in Egr-1 expression relative to sham-operated controls were confirmed by RT-PCR at the linear range of amplification (Fig. 3) and quantified, and the results are presented graphically in Fig. 4. Relative levels of Egr-1 expression increased threefold by 3 h and remained elevated through 24-h ischemia. Although not present on the array, analysis of Egr-3 expression was considered for further examination, as this represents a member of the Egr family (21). Oligonucleotide primers were designed to amplify a gene-specific cDNA fragment of Egr-3 (Fig. 3). Quantification of amplified Egr-3 fragments indicated a significant increase in expression after 3-h ischemia with a maximum increase (5-fold) by 24 h. Lung Kruppel-like factor (LKF) was another transcription factor on the array that appeared to demonstrate altered expression levels (Table 1). However, examination by RT-PCR showed no substantial increase in gene expression levels of LKF at other time periods of ischemia (Fig. 4).

As Egr-1 has been shown to induce cardiac α-MHC in rat cardiomyocytes (9), the pattern of α-MHC expression was also analyzed by RT-PCR. In the represented autoradiogram of the array membrane (Fig. 2), gene expression of α-MHC was beyond the linear range of quantification. Maximum induction in α-MHC expression (Fig. 4) was observed at 1 and 3 days after ischemia compared with controls by RT-PCR analysis. Fetal MLC was another gene representing a cardiac muscle protein whose expression was altered twofold on the array membrane (Table 1). Ischemic induction of MLC expression was supported by RT-PCR analysis (Fig. 4), in which a 60–70% increase in expression was observed 1 and 3 days after ischemia.

An unpredicted observation was a decrease in expression of the cdk4 and cdk6 inhibitor p18ink4 (Table 1). Detectable hybridization signals of other cell cycle regulators on the array included cyclin G (G/M specific) and p21Cip/Waf1, however, altered expression levels due to ischemia were only observed for the CDK inhibitor p18ink4. Further analysis by RT-PCR (Fig. 5) indicated a decreased expression after 0.5 h, 3.0 h, and 24 h of ischemia (80%, 50%, and 20% reduction, respectively). This observation is the first report in which a downregulation of p18ink4 has been associated with ischemia.

Among the apoptotic and other stress-related genes on the array, only transcription of microsomal glutathione S-transferase was altered (Table 1), in which a reduction in expression levels (40–60%) was noticed at the earlier time periods of ischemia (Fig. 5). Examination of expression of apoptosis-related molecules including caspases and Bcl-2 family members showed that these were undetectable on the array (Fig. 2), in contrast to BAG-1 (bcl-2-binding protein with anti-cell death activity), whose transcription levels did not change (Fig. 5). The absence of detectable expression of other apoptotic genes on the array after 24-h ischemia prompted us to examine transcripts of bcl-xL and Bax by RT-PCR analysis (Fig. 6) at other time periods. Both Bcl-2 (anti-apoptotic) and Bax (pro-apoptotic) represent Bcl-related molecules that can form heterodimers with each other. Consequently, relative levels of each molecule may determine the extent of cell death (19), and examination of only one member of this family may provide an incomplete analysis. Densitometry of amplified cDNAs showed a significant increase in the ratios of Bax to bcl-xL amplified fragments after 3 and 7 days of ischemia. This increased ratio was due to an upregulation of Bax levels rather than alterations in bcl-xL gene expression. Altogether, the profile of gene expression on the cDNA array was used for selecting target genes for further analysis.
DISCUSSION

A detailed understanding of the molecular events that accompany myocardial ischemia will enhance the future development of therapeutic strategies for treatment of cardiac injury. Defined time periods of ischemia were examined (0.5 h through 7 days), as an aim of this study was to define the molecular changes that occurred early after the onset of ischemia. A determination of the early insults induced by ischemia may furnish insights in the development of potential therapeutic interventions during an acute time period of ischemia. This study demonstrates the successful adaptation of the surgical procedure of coronary occlusion in a mouse survival model of ischemia as demonstrated by impaired hemodynamic function.

The cDNA array strategy provides an effective means of comparing the molecular profile between two physiological conditions. In principle, this approach provides a sensitive method of simultaneously analyzing the abundance of 588 individual RNA transcripts, which is less laborious than other methods such as Northern blotting or subtractive hybridization assays. The resultant transcription pattern on the cDNA array membranes provided guidance for selecting target genes for further examination. Relative changes in expression levels by array hybridization were confirmed by RT-PCR analysis, except in the case of LKLF, for which no significant increase in gene expression was observed after PCR amplification. Moreover, the hybridization pattern was tissue specific in that robust expression

Fig. 3. RT-PCR transcript analysis of Egr-1 and Egr-3 genes after timed ischemia. Amplification of Egr-1, 18S rRNA, and Egr-3 genes showing the predicted fragment size after separation on a 3.5% NuSieve 3:1 agarose gel and visualization by ethidium bromide staining. The 18S rRNA gene was amplified for 24 cycles, whereas quantification of Egr-1 and Egr-3 expression was done after 28 cycles.
was observed for a cardiac related gene, α-MHC. Cardiac ventricle tissue also produced a predictable tissue restrictive pattern against the Kruppel-like family (KLF) of transcription factors. LKLF (lung KLF) was expressed in moderate levels by heart RNA, whereas the gut (epithelial specific) and erythroid (red cell specific) KLFs (30) were undetectable after stringent hybridization.

Analysis of the transcription pattern showed that only a small number of genes located on the array were affected by coronary artery ligation without reperfusion. It should be noted that the examined expression profile after ischemia is limited by the selection of genes located on the experimental array membrane. As the mouse genome may contain ~140,000 genes, analysis of 588 genes represents only 0.4% of the total genome. Analysis of other commercially available arrays with different selection of genes is likely to yield other cellular targets that are elicited by cardiac ischemia. In addition, our study focused only on genes whose expression changed more than twofold by hybridization analysis, and it is conceivable that genes altered by lower levels may be physiologically relevant.

Myocardial ischemia provoked transcription of genes associated with a fetal program such as p18ink4, previously shown to accumulate during myogenic proliferation and differentiation (22), and genes linked with cardiac muscle development including α-MHC and MLC (17). This study represents one of the first instances in which downregulation of p18ink4 has been implicated with cardiac ischemia, although experimental support for a role of other cell cycle regulators has been shown in vascular ischemia (25) and in myocytes (26). In these instances, ischemia was associated with an increase in cdc2 kinase levels (25) or expression of...
various cyclins (26). In this study, we cannot discern whether alterations in p18ink4 were limited to specific cell populations such as myocytes or fibroblasts.

Overall, most changes appeared to occur within 24 h induction of ischemia, except for alterations in Bax gene expression. Complete coronary ligation (acute phase) led to a decrease in levels of p18ink4 and glutathione S-transferase gene expression that coincided with an increased expression of the Egr transcription factors and myosin-related genes. The observed reduction in expression of microsomal glutathione S-transferase may be indicative of disturbances caused by oxygen deprivation. On the other hand, reperfusion after ischemia in rat hearts caused an increase in glutathione S-transferase enzyme activity as part of an adaptive response to oxidative stress (24). Recently, stimulation of Egr-1 expression has been associated with the induction of fibroblast growth factor during endothelial injury (28). Moreover, stress conditions have been shown to increase transcription of Egr-1 in porcine myocardium after ischemia and reperfusion (3), in the left ventricle of rabbit hearts after application of an intracranial pressure (31) and during ischemia in this study. It is conceivable that induction of Egr-1 may direct the transcription of downstream genes that are associated with either the cardioprotective or degenerative processes associated with ischemia. In this context, the increase in cardiac α-MHC may represent a protective response at 24 to 72 h of ischemia. In other experiments, no change in α-MHC mRNA was observed after longer periods (1–3 wk) of coronary artery ligation in the rat.

Undetectable levels of apoptotic-related genes on the array were somewhat unexpected and may suggest that the transcription program for apoptosis may differ from changes in protein expression observed in rat hearts after ischemia (14, 16). However, results from this study do not prove that gene expression changes may be initially limited to the infract zone. Within 3–7 days after myocardial ischemia, expression alterations in the heart ventricle appear to involve pro-apoptotic genes such as Bax rather than molecules associated with anti-cell death activity such as bcl-xl and BAG-1. These observations are not inconsistent with other studies showing that moderate short-term ischemia did not alter mRNA expression of bcl-xl, Bak, or Fas in the left ventricle of porcine myocardium (1).

In summary, utility of the cDNA array approach allowed a rapid detection of genes associated with cardiac ischemia. This strategy avoids a time-consuming examination of genes whose expression may not be specifically related with ischemia. Moreover, this approach may assist with developing insights into the contributing molecular mechanisms underlying myocardial dysfunction. These advantages as well as the convenience of cDNA array technology are likely to usher its widespread use in the immediate future.

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