Temporal Patterns of Gene Expression After Acute Hindlimb Ischemia in Mice

Insights Into the Genomic Program for Collateral Vessel Development

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OBJECTIVES	We sought to understand the genomic program leading to collateral vessel formation.
BACKGROUND	Recently, technology has advanced to the point that it is now possible to elucidate the large array of genes that must be expressed, as well as the temporal expression pattern, for the development of functionally important collateral vessels. In this investigation, we used deoxyribonucleic acid array expression profiling to determine the time course of differential expression of 12,000 genes after femoral artery ligation in C57BL/6 mice.
METHODS	Ribonucleic acid was extracted from the adductor muscle, which showed no signs of ischemia. Sampling was at baseline, 6 h, and 1, 3, 7, and 14 days after femoral artery ligation or sham operation.
RESULTS	Femoral artery ligation caused the differential expression (>2-fold) of 783 genes at one or multiple time points: 518 were induced and 265 were repressed. Cluster analysis generated four temporal patterns: 1) early upregulated (6 to 24 h)—immediate early transcriptional factors, angiogenesis, inflammation, and stress-related genes; 2) mid-phase upregulated (day 3)—cell cycle and cytoskeletal and inflammatory genes; 3) late upregulated (days 7 to 14)—angiostatic, anti-inflammatory, and extracellular matrix-associated genes; and 4) down-regulated—genes involved in energy metabolism, water channel, and muscle contraction. Microarray data were validated using quantitative reverse transcription polymerase chain reaction.
CONCLUSIONS	This study documents the large number of genes whose differential expression and temporal functional clustering appear to contribute to collateral formation. These results can serve as a genomic model for arteriogenesis and as a database for developing new therapeutic strategies. (J Am Coll Cardiol 2004;43:474–82) © 2004 by the American College of Cardiology Foundation

The development of collateral vessels, referred to as arteriogenesis, is a complex process requiring the action of multiple genes expressed in an appropriate time-dependent manner. The process is undoubtedly tightly regulated by multiple factors, some of which include the appropriately timed expression of both arteriogenic and arteriostatic factors, the relative balance of which changes over the course of time (1).

Understanding the genomic program leading to collateral formation is of fundamental importance to develop insights into what factors are responsible for the highly variable ability that different patients have to form collaterals, as well as optimal therapeutic strategies designed to enhance collateral formation. Despite its importance, little is known about the array of genes that must be expressed, or their temporal expression pattern, for functionally important collaterals to develop.

Recent advances in microarray technology provide the tools to perform comprehensive, quantitative comparisons at the transcriptional level of thousands of genes simultaneously (2). In this regard, a gene chip is commercially available for transcriptional profiling that contains ~12,000 mouse genes. Fortunately, one of the better models commonly used for arteriogenesis research is a murine model of acute hindlimb ischemia. Thus, the animal model and technology are available that make possible sophisticated gene expression studies. Therefore, we investigated the temporal profiles of global gene expression in muscle surrounding developing collaterals during the response to acute hindlimb ischemia in the mouse, with the goal of obtaining a more comprehensive understanding of the genomic program of arteriogenesis.

METHODS

Animal model. Twelve-week-old male C57BL/6 mice were used for the experiments. Animals either underwent left femoral artery ligation or a sham operation. Mice were anesthetized with a mixture of ketamine (40 mg/kg) and xylazine (100 mg/kg). A skin incision was performed on the medial aspect of the left thigh. After careful dissection of the vein and nerve, the femoral artery was ligated and cut immediately distal to the inguinal ligament and proximal to the popliteal bifurcation site (Fig. 1). No branches were

Abbreviations and Acronyms

cDNA	=	complementary deoxyribonucleic acid
ENA-78	=	epithelial neutrophil activating protein-78
HIF1	=	hypoxia-inducible factor-1
Hmox	=	heme oxygenase
HSP	=	heat shock protein
IL	=	interleukin
IP	=	interferon-gamma-inducible protein
MCP1	=	monocyte chemoattractant protein-1
MIG	=	monokine induced by interferon-gamma
MIP	=	macrophage inflammatory protein
MMP12	=	metalloelastase (metalloproteinase-12)
MT1	=	metallothionein-1
RNA	=	ribonucleic acid
RT-PCR	=	reverse transcription-polymerase chain reaction

ligated along the length of the excised segment. In the sham group, the femoral artery was dissected free but not ligated.

After surgery, all animals were closely monitored and sacrificed at five different time points. The entire adductor muscle was used for array studies and Western blotting. The study protocol was approved by the Animal Care and Use Committee of the MedStar Research Institute.

Tissue collection and ribonucleic acid (RNA) preparation. The temporal expression profiles were analyzed at five time points (baseline, 6 h, and 1, 3, 7, and 14 days after surgery), because the majority of flow recovery occurs during this period. Left adductor muscles were harvested from groups subjected to femoral artery ligation and from sham-operated groups (n = 4 animals per group per time point). Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from pools of four mice using Trizol reagent (Invitrogen, Carlsbad, California), according to the manufacturer's instructions. The RNA was cleaned using a RNeasy mini-kit (Qiagen, Va-
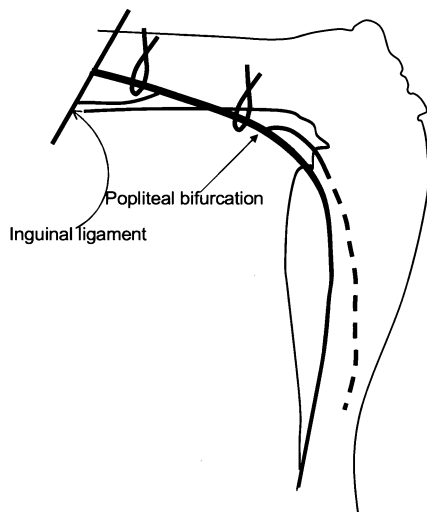

Figure 1. Mouse hindlimb model. Schematic drawing of the femoral artery ligation sites. The femoral artery was ligated and cut immediately distal to the inguinal ligament and proximal to the popliteal bifurcation site. The entire adductor muscle was used for array studies and Western blotting.

lencia, California) and stored at −80°C. The RNA concentrations were obtained by measuring absorbance at 260 nm, and its integrity was verified with a 2% agarose mini-gel.

Microarray analysis. Double-stranded complementary deoxyribonucleic acid (cDNA) was synthesized from 8 μg total RNA. For the first cDNA strand synthesis, oligo(dT) primers were annealed to the RNA, and extension by reverse transcriptase was performed in the presence of deoxyoligonucleotides. The second strand was synthesized using deoxyribonucleic acid polymerase I and purified using phase-lock gels-phenol/chloroform extraction, followed by ethanol precipitation.

In vitro transcription, using double-stranded cDNA as a template in the presence of biotin-labeled ribonucleotides, was performed by using an Enzo in vitro transcription kit (Enzo Diagnostics, Inc., Farmingdale, New York). Biotin-labeled complementary RNA was purified, fragmented, and hybridized to Affymetrix Murine Genome U74Av2 chips (Affymetrix, Santa Clara, California), which contains ~12,000 mouse genes. Hybridization, washing, antibody amplification, staining, and scanning of probe arrays were performed according to the Affymetrix Technical Manual.

Scanned raw data were processed with Affymetrix Gene-Chip version 5.0 software. The average intensity value for each probe set, which directly correlates to messenger RNA abundance, was calculated as an average of fluorescence differences for each perfectly matched probe versus single-nucleotide-mismatched probe. This software also gives each gene a qualitative assessment of "absent" or "present" calls. Data sets on each GeneChip were normalized by scaling total chip fluorescence intensities to a common value of 800 before comparison. All data were imported into GeneSpring version 5.0 software (Silicon Genetics, Redwood City, California) for further analysis. The fold changes of each gene at different time points were calculated based on the normalized values and represented as relative to baseline (day 0). We selected the genes with a >2-fold change over baseline in at least one time point. A value of differential gene expression >2-fold at one or multiple time points between the femoral artery ligation and sham-operated groups was considered as significant. Genes that were induced or repressed at similar levels in both groups were excluded from the analysis. Cluster analysis was performed to identify families of genes with a similar time-dependent expression.

Real-time reverse transcription polymerase chain reaction (RT-PCR). To validate the microarray data, expression of three selected genes (monocyte chemoattractant protein-1 [MCP1], metallothionein-1 [MT1], and metalloelastase [or metalloproteinase; MMP12]) were analyzed using quantitative real-time RT-PCR. Complementary DNA was synthesized from 200 ng total RNA in a 20-μl reaction using TaqMan reverse transcription reagents (Applied Biosystems, Branchburg, New Jersey). Real-time RT-PCR was performed with an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied

Table 1. Representative Genes Differentially Regulated Between Mice With Femoral Artery Ligation and Sham Control Operation

Gene	Accession Number	Femoral Artery Ligation					Sham Operation				
		6 h	1 d	3 d	7 d	14 d	6 h	1 d	3 d	7 d	14 d
Angiogenesis											
Cyr61	M32470	3.10	2.04	3.03	3.01	1.66	1.28	2.73	2.89	2.51	
HDGF	D63707	2.97	2.01	2.67	1.94	1.41		2.09			
IP10	M33266		2.69	2.89	4.10	2.57		3.17	3.16	1.64	
MCP1	M19681	9.94	28.8	12.5	3.61	2.61	7.67	10.8	6.11	1.90	
MIG	M34815		1.25	0.88	2.77	0.81			0.83	0.72	
MMP12	M82831			5.44	18.0	58.9				6.21	2.06
PIGF	X80171		3.46	1.76							1.94
TGFβ1	AJ009862				15.7	21.7					
Cell Growth and Survival											
Ccnb1-rs1	X64713			53.8	19.5				31.3		
Cdc2a	M38724			4.12	2.61				1.97		
Cdkn1a	AW048937	3.03	5.38	2.36	3.45	2.48	2.78	2.30	1.72	1.72	
Lcn2	X81627	2.56	60.6	10.2	0.97	1.02	5.46	8.64	5.57		
Mki67	X82786			2.81	2.62	1.70			1.34		
Mts1	M36579	0.73	3.50	7.10	3.79	3.75	1.18	4.83	3.46	3.30	1.19
Hmox1	X56824	3.23	8.52	5.21	1.58	1.71	5.34	2.85	3.29	1.24	0.96
HSP70-3	M12571	2.11	5.36	0.67	0.95	0.79	1.01	1.07	0.69	0.75	0.62
Mt1	V00835	8.14	40.8	20.4	1.94	0.76	17.9	11.37	17.4	0.79	
SGP-1	AF037437	42.1		29.5	27.4	47.6	59.6				
Zac1	X9504			4.26	77.8	58.7				14.4	
Cell Shape and Motility											
Crp2	D88792			3.69	8.74	5.13		2.80	3.32	4.38	
Lmnb1	M35253	3.52	2.23	3.62	2.41				1.08	1.68	
Myhse	M74753			1.79	54.0	9.46			2.99	3.33	
Myh8	M12289		1.61	1.15	24.1	11.1	2.29	1.49	1.47	3.54	2.64
Myla	M19436		0.81	1.43	32.6	14.3		1.08	2.87	6.76	1.62
Tubb5	X04663	0.86	2.14	3.53	2.72	2.12	0.82	1.77	2.22	1.48	0.71
Mlc2	M91602	0.63	0.55	0.04	0.07	0.12	0.50	0.94	0.12	0.46	0.29
Myhcb	AJ223362	0.67	0.73	0.11	0.11	0.19	0.63	1.46	0.30	0.61	0.42
Tpm5	U04541	0.64	0.74	0.14	0.14	0.18	0.55	1.14	0.33	0.55	0.35
Cytokines and Inflammation											
CD14	X13333	2.33	8.23	2.53	2.56	2.25	2.04	3.29	1.91	1.38	
CD68	X68273		2.47	5.05	4.41	3.23		2.41	3.06	1.34	
ENA78	U27267	9.57	266	36.6		9.10	27.4	60.7	34.3		
Gp49	M65027	2.29	14.9	6.42	5.23	4.89	1.69	4.98	3.28	2.22	0.91
Gp49b	U05265	2.53	9.81	4.25	3.78	3.76	3.08	5.55	2.71	1.60	
Lcp2	U20159		2.57	3.69	2.16	1.13	1.02	1.63	3.63	1.07	
IL1β	M15131		240	49.9				107	50.8		
IL6	X54542	255	211	161			114	150	161		
Lipo1	M69260	0.82	1.44	1.98	2.14	2.69	0.69	1.84	1.90	1.84	0.81
MCP3	X70058	4.96	34.5	15.2	4.33	2.36	5.49	18.28	7.55	2.12	0.44
MIP1α	J04491		6.75								
MIP2	X53798	52.3	394	24.9	6.43	31.8	28.2	112	24.1		
MRP8	M83218	2.01	10.3	2.74	0.53	1.04	1.12	3.49	4.30	0.14	
Extracellular Matrix											
Bgn	X53928	0.99	1.12	2.71	6.92	4.70	0.78	1.59	1.71	3.19	1.09
Bmp1	AA518586	2.38	1.57	1.87	2.38	3.11				1.57	1.87
Ctss	AJ223208	0.25	1.47	5.17	5.38	4.35	0.32	2.35	3.24	1.93	0.52
Coll1a2	X58251	0.95	1.10	2.10	6.35	7.35	0.90	1.26	1.73	3.97	1.14
Coll3a1	AA655199	1.49	1.20	5.30	9.94	12.8	0.69	2.38	5.28	7.42	1.93

Table 1. Continued

Gene	Accession Number	Femoral Artery Ligation					Sham Operation				
		6 h	1 d	3 d	7 d	14 d	6 h	1 d	3 d	7 d	14 d
CPX-1	AF07773			18.0	19.9	15.0					
Lgmn	AJ000990	1.17	2.99	5.58	6.49	4.40	0.88	2.98	5.20	2.07	1.22
Mglap	D00613	0.81	1.52	1.24	4.21	2.65	0.62	1.75	1.26	1.65	0.61
MMP3	X66402		8.30	7.86	3.30	5.08		9.40	14.3	3.67	
MMP13	X66473			1.78	11.6	8.15					
MMP14	AF022432	1.05	0.84	1.35	2.55	3.15	1.14	1.26	1.16	1.85	1.21
OSF-2	D13664	0.94	1.22	3.12	25.9	18.2	0.52	1.35	2.89	10.7	1.26
PAI-1	M33960	3.34	5.57	2.13	2.30	1.33	2.7	1.36	1.92	1.25	0.71
Spp1	X13986		11.1	32.7	14.6	19.5		10.8	9.45	2.81	
Tnc	X56304	1.65	3.35	2.99	19.85	8.78		1.64	4.36	3.82	
Metabolism											
Abca1	X75926			2.46	3.23	2.57		1.67	1.54	2.66	
Acadm	U07159	0.86	0.83	0.48	0.46	0.56	0.81	1.10	0.56	0.92	0.72
Apoe	D00466	0.67	0.83	2.58	5.90	4.17	0.61	0.94	2.81	2.79	1.26
Cox8b	U15541	0.62	0.76	0.33	0.46	0.36	0.84	1.13	0.54	1.05	0.68
Cyp1b1	X78445		3.03	3.22	2.51	2.01		2.36	3.29		
Glut-4	M23383	0.98	0.54	0.43	0.35	0.46	1.11	0.70	0.48	0.85	0.90
Lp1	AA726364	0.56	0.65	0.41	0.42	0.42	0.52	1.0	0.85	0.69	0.49
Pdha-1	M76727	0.68	0.88	0.34	0.43	0.48	0.70	0.62	0.53	0.95	0.61
Signaling											
Activin	X69620		21.2	3.54							
Adam8	X13335		60.0	40.0	18.7			25.0			
Bit	D85785			12.4	13.2	13.6					
CD116	M85078		6.81	6.27	8.47	9.24		4.51			
Emr1	X93328			5.10	6.61	2.71		2.62	4.34		
Fkbp10	L07063	2.25	1.76	2.43	3.69	3.53		1.80	2.03	2.05	
Gna12	M63659			2.80	4.03	3.38					
Itgax	AI035495				3.81	3.54					
Pld3	AF02612			2.10	3.56	2.23					
Wisp1	AF10077			2.01	8.70	4.15			2.16	2.47	
Transcription											
Alrp	AF041847	0.90	36.3	16.6	12.8	2.45	4.05	8.93	19.6	3.83	0.62
c-myc	L00039	2.61	6.71	3.09	2.20	0.88	2.67	2.38	3.91	1.64	
Fos	V00727	17.9	12.8	8.15	6.38	3.29	5.68	10.5	13.1	16.1	2.51
Junb	U20735	139	74.7	44.7	27.4		140	31.4	24.3		
Ler2	M59821	3.06	2.70	1.62	1.46	0.96	1.47	1.55	1.71	1.74	0.70
Ncoa1	U64828	2.63	0.99	1.10	1.32	1.61		1.37	1.32	1.5	
Rnf4	AV37235	12.1									
Other Functions											
Aq1	L02914	0.99	0.97	0.82	0.49	0.59	1.71	1.14	0.83	1.06	1.07
Aqp4	U88623	0.78	0.09	0.13	0.12	0.39	0.59	0.11	0.17	0.57	0.43

Biosystems Inc., Foster City, California), according to the manufacturer's recommendation. The principle of real-time RT-PCR has been described in detail elsewhere (3). Primer and probe sequences were chosen using sequences in the GenBank: MCP1 forward (5′-GAGCATCCACG-TGTTGGCT-3′), reverse (5′-TGGTGAATGAGT-AGCAGCAGGT-3′), probe ([6-FAM]-AGCCAGAT-GCAGTTAACGCCCCACT-[TAMRA-FAM]); MT1 forward (5′-CCTGCTCCACCGGCG-3′), reverse (5′-GC-AGACACAGCCCTGGG-3′), probe ([6-FAM]-CTG-CTGCTCCTGCTGTCCCGTGTG-[TAMRA-FAM]; and MMP12 forward (5′-GAGGCAGAAACGT-

GGACTAAAAGT-3′), reverse (5′-GTTCATGAA-CAGCAACAAGGAAGA-3′), probe [6-FAM]-TTT-CAAGGCACAAACC-[TAMRA-FAM]). Quantitative PCR was performed in 96 sample plates. The cDNA equivalent of 100 ng total RNA/tube containing TaqMan PCR Universal Master Mix (Applied Biosystems), 100 nmol/l probe, and 200 nmol/l of each primer was used. As a control for RNA integrity and for assay normalization, 18S ribosomal RNA was amplified using a TaqMan ribosomal RNA control reagents kit (Applied Biosystems). Gene expression levels were compared using the C_t method, as follows: ΔC_{t1} = [C_t of target gene in RNA from ligated mice] − [C_t of 18S in RNA

from ligated mice]; and $\Delta C_{t2} = [C_t$ of target gene in RNA from sham mice] $- [C_t$ of 18S in RNA from sham mice]. Expression levels were calculated by $2^{\exp(-[\Delta Ct1 - \Delta Ct2])}$.

Western blot analysis of tissue hypoxia-inducible factor (HIF)1-alpha levels. Adductor and calf muscles were lysed in ice-cold buffer, and 40 μg protein was separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transferring onto nitrocellulose membrane, the blots were incubated with a goat anti-mouse HIF1-alpha antibody (Santa Cruz Biotechnology, Santa Cruz, California) and visualized with enhanced chemiluminescence (Pierce, Rockford, Illinois).

RESULTS

A total of 783 genes showed at least twofold differential expression between the femoral artery ligation and sham groups at one or multiple time points: 518 were induced and 265 were repressed. The largest number of differentially regulated genes was observed seven days after surgery; representative genes are shown in Table 1. Complete lists of upregulated and downregulated genes are presented as data supplements. (For Appendices 1 and 2, please see the February 4, 2004, issue of *JACC* at www.cardiosource.com/jacc.html.) Genes were classified into eight groups according to their predominant function, although some genes were difficult to classify because they exhibit multiple dominant functions.

Differential gene expression according to function. A number of *angiogenesis-related genes*, including cysteine-rich protein-61, hepatoma-derived growth factor, MCP1, placental growth factor, and transforming growth factor-beta, were differentially upregulated in the femoral artery ligation group as compared with the sham group (Table 1). However, some genes known to be related to angiogenesis (angiopoietin 1, fibroblast growth factor 1, endothelial nitric oxide synthase, FMS-related tyrosine kinase 1, hypoxia inducible factor 1 alpha, tyrosine kinase with Ig and EGF homology domains, and vascular endothelial growth factor B and C) were undetectable or not significantly changed, and other such genes (vascular endothelial growth factor A and D) were similarly upregulated in both groups.

It is interesting to note a significant upregulation of *genes thought to exert angiostatic activities* (interferon-gamma–inducible protein-10, monokine induced by interferon-gamma [MIG], and MMP12) during the late time points after femoral artery ligation. Stress-related genes, including heme oxygenase-1 (Hmox), heat shock protein (HSP)70-3, and MT1, were highly upregulated during the early time points.

Inflammatory response-related genes comprised the largest gene cluster that was upregulated. Although upregulated in both groups, the genes exhibited higher expression levels for longer times in the femoral artery ligation versus sham group. The epithelial neutrophil activating protein (ENA)-78 profile showed a peak level at one day after

surgery. Interleukin (IL)-1-beta, IL-6, MCP1, macrophage inflammatory protein (MIP)1-alpha, and MIP2 expression also demonstrated early peak levels after femoral artery ligation, and then slowly declined. Markers for inflammatory cell infiltration were also detected: neutrophil markers (CD14, MRP8) appearing at 6 h, followed by those for macrophages (CD14, CD68), mast cells (Gp49, Gp49b), and lymphocytes (Lcp2). The most highly expressed cytokines (>10-fold induction) were ENA-78, IL-1-beta, IL-6, MCP1, MCP-3, and MIP2.

Cytoskeletal genes, including cysteine-rich protein 2 (double LIM protein-1) and tubulin were slowly induced and remained at high levels throughout the study period. *Genes encoding contractile proteins* were either enhanced (Myhse, Myh8, Myla) or repressed (Mlc2, Tpm5).

The expression of *extracellular matrix-associated genes* occurred at late time points. The genes in this category included those encoding several types of collagen, biglycan, Ctss (cathepsin S), Mglap (matrix Gla protein), and matrix metalloproteinases (MMP-3, MMP12, MMP14). The genes encoding CPX-1 (metallocarboxypeptidase), osteoblastic-specific factor-2, and Spp1 (osteopontin) manifest similar time trends of expression.

Genes encoding proteins involved in energy metabolism (Acadm, Cox8b, Glut-4, Lp1, Pdha-1) were generally repressed across the time course of the experiment, whereas the *genes involved in cholesterol transport* (ABCA1 and ApoE) were slightly upregulated. There were a number of genes corresponding to expressed sequence tags that were differentially expressed (data not shown).

Clustering gene expression patterns. The expression profiles were clustered according to similarity of temporal expression patterns, using *k*-means cluster analysis (Fig. 2).

EARLY INDUCTION. Genes that were rapidly induced at early phase (peak levels at 6 to 24 h) included immediate early transcriptional factors (Fos, Junb, Ler2), followed by genes associated with angiogenesis and inflammation, as well as stress-related genes.

MID-PHASE INDUCTION. Genes that were upregulated at mid-phase, defined as those genes whose peak expression occurred at day 3, included genes associated with cell cycle regulation, cytoskeletal-related genes, and additional inflammation-related genes.

LATE-PHASE INDUCTION. Genes that were upregulated at the late phase, defined as those genes whose peak expression occurred at days 7 to 14, included genes associated with angiostatic, anti-inflammatory (lipo1), cytoskeletal, and extracellular matrix-associated effects.

DOWNREGULATION. Genes that were downregulated (<0.5-fold) after surgery included those genes involved in energy metabolism, water channels (Aq1, Aqp4), and muscle contractions.
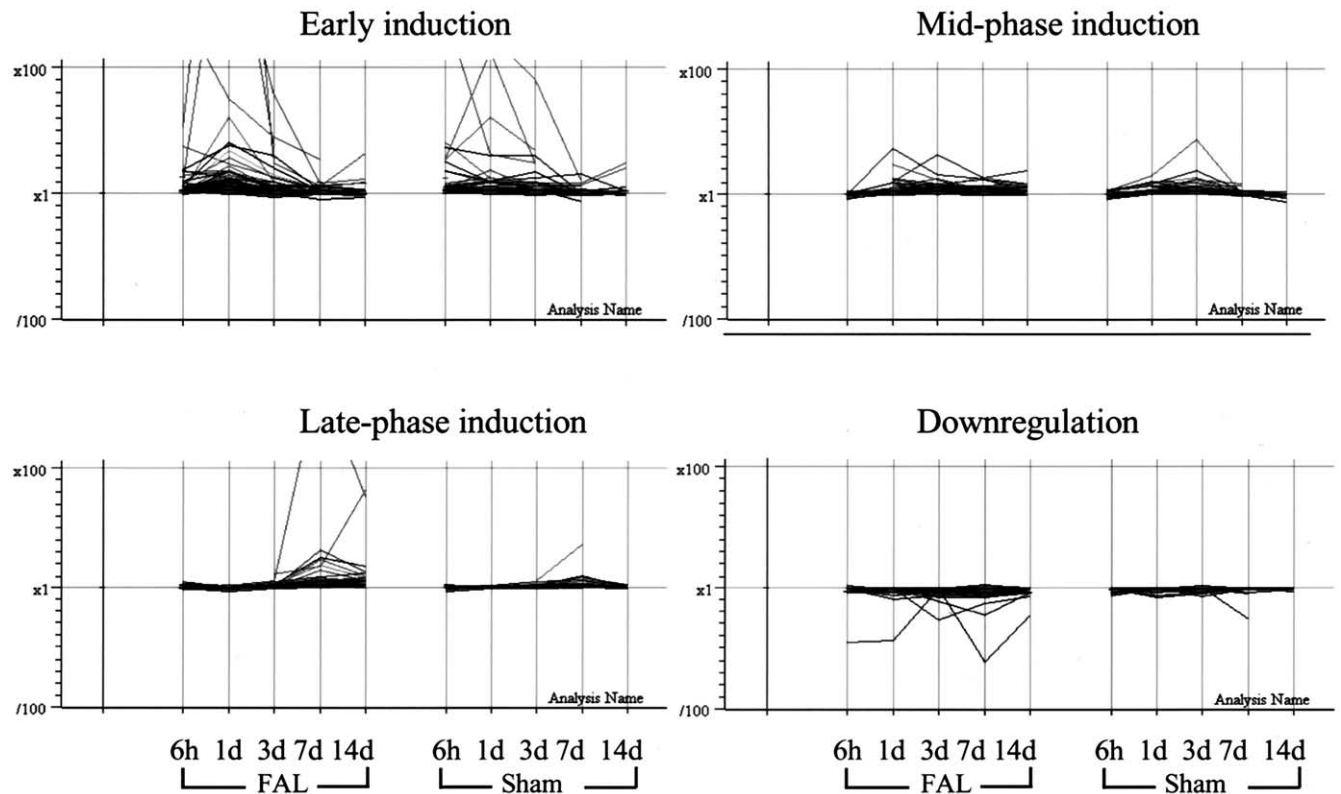
Figure 2. Expression profiles clustered according to similarity of temporal expression patterns, using *k*-means cluster analysis. Early induction includes upregulated genes whose peak expression occurred at 6 to 24 h after ligation. Mid-phase induction includes upregulated genes whose peak expression occurred at day 3. Late-phase induction includes upregulated genes whose peak expression occurred at days 7 to 14. Downregulation includes genes whose expression was downregulated (<0.5-fold). FAL = femoral artery ligation.

Validation by real-time RT-PCR. Table 2 summarizes the comparison of fold changes in expression for three representative genes after surgery, showing consistent changes between TaqMan quantitative PCR and microarray analysis.

Western blot analysis of tissue HIF1-alpha levels. The HIF1-alpha protein was examined in the adductor and calf muscles (Fig. 3). In calf muscle, HIF1-alpha protein levels were elevated during the first three days after femoral artery ligation, whereas there was no evidence of HIF1-alpha expression through the study period in adductor muscle.

DISCUSSION

In this investigation, we have identified dynamic global changes of gene expression that occur in a region of active

Table 2. Changes of Three Selected Genes Validated by Real-Time RT-PCR (MCP1, MT1 at Day 1, and MMP12 at Day 7)

Accession No.	Gene	Relative Ratios (Femoral Artery Ligation/Sham)	
		Microarray	RT-PCR
M19681	MCP1	2.6	3.9
V00835	MT1	3.6	4.2
M82831	MMP12	2.9	3.2

MCP1 = monocyte chemoattractant protein-1; MMP12 = metalloelastase (metalloproteinase-12); MT1 = metallothionein-1; RT-PCR = reverse transcription-polymerase chain reaction.

collateral growth after induction of ischemia caused by femoral artery ligation. Compared with a sham-operated group, a total of 783 genes in the femoral artery ligation group changed expression levels by at least two-fold at one or multiple time points. Of course, we cannot be certain that all of these genes are related to collateral development and what portion of them are critical components of the response. Furthermore, it is also possible that some of the changes in gene expression we found resulted from a response to ischemia that has nothing to do with collateral development.

To further understand the potential role of ischemia in inducing the gene expression changes we found, we characterized the ischemic profile of the adductor muscle, the tissue used for RNA extraction. This was accomplished by analyzing the tissue for protein levels of HIF1-alpha, which is mainly regulated post-translationally and is a critically important cell sensor of hypoxia (4). Under normoxic conditions, HIF1-alpha protein is ubiquitinated, which targets the molecule for proteasome-mediated degradation. When the cell is exposed to hypoxia, ubiquitination no longer occurs, and levels of HIF1-alpha protein are stabilized. Therefore, tissue levels of HIF1-alpha provide a marker of hypoxia/ischemia. Using this marker, we found that HIF1-alpha protein was not detectable in the adductor muscle, which lies proximally in the thigh. However,
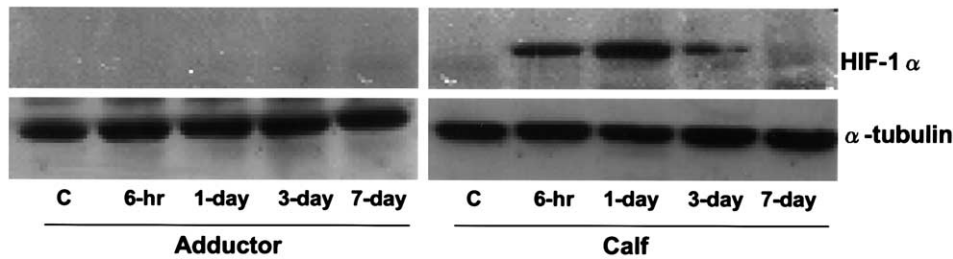
Figure 3. Time course of hypoxia-inducible factor-1 (HIF-1) alpha expression in adductor and calf muscles after femoral artery ligation. C = control.

HIF1-alpha was elevated in the calf muscle during the first three days after femoral artery ligation. In addition, HIF1-alpha target genes relating to anaerobic energy metabolism (aldolase A, enolase 1, lactate dehydrogenase 1, phosphofructokinase, and phosphoglycerate kinase 1) showed no difference in expression in the adductor muscle between the sham and ischemic groups (data not shown). Finally, Western blotting showed no change in LDH1 gene expression in the adductor muscle, compared with significant induction of LDH1 in calf muscle after femoral artery ligation (data not shown).

Thus, the tissue we used for transcriptional profiling and in which collaterals are developing demonstrated no overt evidence of ischemia. These findings indicate that ischemia plays either no or at most a minor role in the gene expression results we obtained. These results also support an interesting previously described finding—that collateral vessels developing proximal to an arterial obstruction do not require the local expression of either VEGF or HIF1 (5).

It is important to emphasize that our model is one in which collateral development has been well documented. In the mouse model of hindlimb ischemia, immediately after femoral artery ligation, a dramatic reduction in distal hindlimb blood flow occurs, which progressively recovers over time (6). These findings have been observed using laser Doppler perfusion imaging to assess hindlimb blood flow perfusion in the distal hindlimb and are well correlated with microsphere-assessed perfusion, as recently reported (7). Thus, an increase in flow after femoral artery ligation

occurs, and this increase in flow can only be due to an increase in collaterals. In this regard, in the same report, it was found that in the upper thigh, the number and size of second- and third-generation collateral branch arteries progressively increase after femoral artery ligation, changes that correlate with laser Doppler perfusion imaging-assessed flow. Moreover, in a recent a report from our laboratory (8), we evaluated the number of collaterals present within the upper thigh (between muscle bundles and fibers) of wild-type C57BL/6 at baseline and 28 days after femoral artery ligation (same model as in the present investigation). A significant increase in the density (number/surface area) of collaterals above baseline was observed in the operated leg of C57BL/6 mice (increase of 50%). Finally, Scholz et al. (9) demonstrated that the adductor muscle is a site of active arterial remodeling. Thus, we believe there exists compelling evidence clearly indicating that collateral vessels develop in the tissue we are examining for differential gene expression relating to collateral development (the adductor muscle), as shown by laser Doppler perfusion imaging for collateral perfusion, anatomic evidence of the presence of collaterals by several different techniques, and microsphere flow determinations of collateral flow.

Overall, temporal patterns of gene expression after femoral artery ligation can be schematically summarized as shown in Figure 4. It is interesting to note the apparently prominent role played by genes modulating inflammatory responses. We found numerous genes relating to inflammatory responses differentially regulated, with many showing extremely high levels of transcriptional activity. The ENA-78, indicative of early neutrophil infiltration (10), was upregulated early after ischemia onset. Maximum levels of MCP1, which recruits monocytes, were reached by day 1 and slowly declined thereafter. The interferon-gamma-inducible protein-10 and MIG are T-cell attractants, and their expression peaked at day 7. The time course of chemokine expression roughly correlated with the recruited leukocyte markers, consistent with a previous report that chemokines are sequentially expressed during wound healing (10). Interestingly, markers for mast cells (GP49, GP49b) were also detected, supporting a role for these cells in arteriogenesis (11). The anti-inflammatory lipocortin-1 gene was induced late after femoral artery ligation, which probably contributes to resolution of inflammation (12).

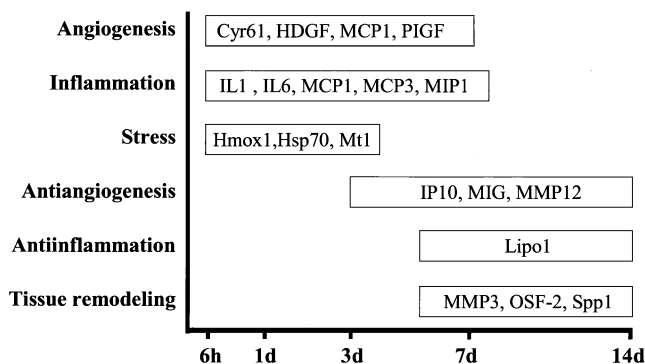Although these finding relating to inflammation could be

Figure 4. Schematic summary of the time course of expression of selected groups of genes after femoral artery ligation. IL = interleukin; IP = interferon-gamma-inducible protein; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; MMP = metalloelastase (metalloproteinase).

an artifact of our specific model, the results support the increasing evidence suggesting that components of inflammatory responses constitute critically important players in the development of collateral vessels (13,14). Our results are not only compatible with such a concept, but also the diversity of the response demonstrates the extraordinarily complex gene responses that are involved in arteriogenesis. Moreover, the sequential expression of pro-inflammatory followed by anti-inflammatory genes suggests that inflammation contributes to early processes, leading to the initiation of collateral formation, but resolves soon after induction begins.

One of the mechanistically important findings of this study which has not been previously described is the increased expression of angiostatic genes (including IP-10, MIG, and MMP12) (15,16) at late time points after arterial ligation. The MMP12 is the most efficient angiostatin-inducing MMP, and angiostatin is one of the most potent angiostatic factors (16). The relatively late appearance of IP-10, MIG, and MMP12 probably contributes to a late shift in the processes involved in arteriogenesis, possibly ushering in a different phase of collateral formation, such as a phase relating to collateral maturation.

Endothelial cell survival is thought to be an essential mechanism during angiogenesis (17). We found that genes involved in cytoprotection signaling (Hmox1, HSP70-3, MT1) were highly upregulated early after femoral artery ligation, indicating that ischemic stress may trigger a genetic program for cell survival. In addition, evidence suggests that Hmox1 and MT1 are involved in angiogenesis (18,19), a conclusion compatible with our finding of the persistent expression of such genes into the mid-phase of gene expression after femoral artery ligation. Taken together, Hmox1 and MT1 may affect endothelial cell survival in collateral-forming tissues and contribute to arteriogenesis.

A number of genes associated with cell structure and extracellular matrix modification are differentially expressed during the late time points, as were several isoforms of collagen, cathepsin S, MMPs, osteoblastic-specific factor-2, and osteopontin. These genes may contribute to collateral development in that an altered balance between extracellular proteolysis and antiproteolysis is associated with growing collateral vessels (20).

Interestingly, aquaporins (Aq1, Aqp4) were significantly repressed throughout the study period. Aquaporins are water channels that regulate transcellular water permeability and have been implicated in the process of edema formation during inflammation (21). In our model, Aqp4 repression may help to prevent excessive edema in the collateral-forming tissues. However, their specific role in arteriogenesis remains unknown.

Finally, in the sham group, inflammation-related genes were also induced after surgery, suggesting that the sham operation itself can induce a number of changes in gene expression. This finding highlights the importance of carefully considering the optimal controls in gene expression profiling studies.

Study limitations. A potential limitation of the study needs to be addressed. Specifically, the adductor muscle, which contains the proximal portions of the developing collateral vessels, was used for RNA extraction. The specimen necessarily contains a far greater proportion of skeletal muscle tissue compared with collateral tissue. Therefore, we cannot at this time definitively distinguish between collateral-related differential gene expression and differential gene expression that relates exclusively to the wound healing response to our intervention.

Despite these limitations, we believe our study provides a preliminary picture of global temporal patterns of gene expression involved in the complex processes contributing to collateral formation. These findings can serve as a genomic model for a more complete understanding of arteriogenesis and as a data base for the development of new therapeutic strategies.

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APPENDIX

For Appendices 1 and 2, please see the February 4, 2004, issue of *JACC* at www.cardiosource.com/jacc.html.