Time course of skeletal muscle repair and gene expression following acute hind limb ischemia in mice

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Paoni, Nicholas F., Franklin Peale, Fay Wang, Carol Errett-Baroncini, Hope Steinmetz, Karen Toy, Wei Bai, P. Mickey Williams, Stuart Bunting, Mary E. Gerritsen, and Lyn Powell-Braxton. Time course of skeletal muscle repair and gene expression following acute hind limb ischemia in mice. Physiol Genomics 11: 263–272, 2002. First published October 22, 2002; 10.1152/physiolgenomics.00110.2002.—DNA microarrays were used to measure the time course of gene expression during skeletal muscle damage and regeneration in mice following femoral artery ligation (FAL). We found 1,289 known sequences were differentially expressed between the FAL and control groups. Gene expression peaked on day 3, and the functional cluster “inflammation” contained the greatest number of genes. Muscle function was depressed for 3 days postligation, but returned to normal by day 7. Decreased muscle function was accompanied by reduced expression of genes involved in mitochondrial energy production, muscle contraction, and calcium handling. The induction of MyoD on day 1 denoted the beginning of muscle regeneration and was followed by the reemergence of the embryonic forms of muscle contractile proteins, which peaked at day 7. Transcriptional analysis indicated that the ischemic skeletal muscle may transition through a functional adaptation stage with recovery of contractile force prior to full regeneration. Several members of the insulin-like growth factor axis were coordinately induced in a time frame consistent with their playing a role in the regenerative process.

expression profiling; insulin-like growth factor; peripheral artery disease; muscle regeneration; inflammation

METHODS

Femoral artery ligation. All animal use protocols were approved by the Genentech Institutional Animal Care and Use Committee. FAL was performed on 8- to 10-wk-old, male C57BL/6 mice (Charles River) under isoflurane (Fort Dodge Labs, Fort Dodge, IA) inhalation anesthesia. Mice were placed in dorsal recumbency, and the ventral skin over the left superficial femoral artery was shaved and prepared for sterile surgery. An incision through the skin was made over the femoral artery. The femoral artery was isolated at the level of the inguinal ligament and ligated using 7-0 silk suture. The wound was closed using 4-0 silk suture (Look, Reading, PA) and a single 7.5-mm Michel wound clip (California Surgical, Hayward, CA). Animals were allowed to recover on a warm-water heating pad until ambulatory. Sham surgery was performed on control mice as described above except that the femoral artery was not ligated. All animals were kept on 12-h light cycle and standard rodent chow.

Muscle stimulation. The twitch tensions produced in response to electrical stimulation were used as a measure of muscle function in an intact animal. The method was a modification of the one reported by Walder et al. (23). Briefly, animals were anesthetized using 2.5% isoflurane in oxygen and maintained at 37°C using a heat lamp. The right sciatic nerve was severed to prevent retrograde stimulation, then the right leg was immobilized with 4-0 silk suture binding the right tibia to a stainless steel platform. The third right
metatarsal was sutured to a Biopac TSD 105A force transducer using 4-0 silk. Two Grass E2 platinum subdermal needle electrodes were placed through the gastrocnemius muscle separated by a distance of 5 mm. A 5-V direct current square wave with duration of 5 ms and an interval of 500 ms was passed through the electrodes using a Grass S8800 stimulator and SIU5 isolation unit. The animal was stimulated for 10 min. Tension from the force transducer was collected and plotted using a Biopac UM100A system with Acknowledge software on an Apple Macintosh computer. A total of 6–10 animals were analyzed per time point. Both peak-force generated and force at the end of 10 min of stimulation (the plateau) were calculated. The time points studied in the assay were time 0 and 4 h, 3 days, 7 days, 14 days, and 28 days post-FAL or sham surgery. The time 0 animals did not undergo any surgical procedures, and thus they represent a baseline value to which all other time points in the sham and ligated groups were compared.

Gene expression analysis. At each time point (15 min (time 0), 4 h, 1 day, 3 days, 7 days, 10 days, 14 days, and 28 days) postsurgery 6 sham and 6 ligated mice were euthanized using CO2. For each animal the posterior calf muscles (gastrocnemius, soleus, and plantaris) were collected in a single tube and immediately frozen in liquid nitrogen before storage at −80°C. Total RNA was extracted from the combined muscle tissues using the RNeasy Maxi Kit (Qiagen). Affymetrix GeneChip probe array analyses were used to identify differentially expressed genes. The method has been described in detail elsewhere (12). Briefly, the total RNA prepared from the six sham and six ligated animals were individually hybridized to mouse A and B Affymetrix GeneChip probe arrays (Affymetrix, Santa Clara, CA) for each time point. To identify differentially expressed transcripts, pairwise comparison analyses were carried out with GeneChip Analysis Suite 3.2. Each of the 6 ligated samples were compared with each of the 6 sham samples, resulting in 36 pairwise comparisons. The genes were ranked according to concordance in the pairwise comparison, and the Mann-Whitney pairwise comparison test was used to calculate significance. Genes where the concordance was ≥80.6% (P ≤ 0.05) at any time point were included in the gene list shown in online Supplemental Tables 1 and 2. An estimate of the fold induction/repression of the genes in the ligated group relative to the sham controls is shown for time points that meet the criterion described above. We have previously validated this approach to identifying differentially expressed genes by confirming with real-time, RT-PCR a relatively large data set generated in this manner (12). In this study selected genes were also confirmed by real-time, RT-PCR using the TaqMan model 7700 sequence detector (ABI). The procedure has been previously described (12). Expression values were normalized to total RNA and expressed as means ± SE. For the purpose of graphic representation in the figures, induced/repressed genes are denoted with a color coding system generated in GeneSpring (Silicon Graphics, Santa Clara, CA) using average difference data from the Affymetrix GeneChip probe arrays.

RESULTS

Histological findings. The images in Fig. 1 illustrate the effect of FAL on soleus muscle tissue in the calf of a young C57 mouse. In control muscle, the myocytes are relatively angular, with peripheral nuclei. Twenty-four hours after ligation, there is cellular swelling, focal necrosis, and interstitial edema. Edema can be hard to detect reliably in histological sections, but it was confirmed independently by measuring a 20% gain in muscle weight at 24 h.

By 3 days postligation, there are densely cellular areas in the most ischemic tissue. In these areas, myocytes are markedly shrunken and are surrounded by proliferating satellite cells, which will differentiate and fuse into mature myocytes. There are also infiltrating inflammatory cells, predominantly macrophages, and proliferating stromal cells. Tissue edema is reduced compared with 24 h.

By 7 days, the affected muscle has a more homogeneous appearance, with all myocytes showing hypertrophic centralized nuclei, and many fibers are markedly shrunken. The macrophage infiltrate has resolved.

At 14 and 28 days, the microscopic changes in muscle appearance gradually resolve as the tissue returns almost to its normal state. Thus FAL induces a transient ischemic injury, affecting a large part of the lower leg. Similar changes occur only to a very limited extent in the thigh.

Muscle function analysis. A typical graph of the force response in a nonligated leg is shown in Fig. 2A. Both the peak force generated and the force at plateau were
significantly reduced from the time 0 baseline value in ligated animals at the 4 h and day 3 time points (Fig. 2, B and C) but returned essentially to normal by 7 days. There was no significant difference in any parameter between sham animals and baseline measurements at any time point (data not shown).

Gene expression analysis. We found that 1,289 known sequences were differentially expressed between ligated animals and sham-operated controls at one or more time points post-FAL; 962 were induced, and 327 were repressed. No changes in gene expression were found for two of the time points, time 0 and 28 days. Also, no genes were significantly repressed at the 4 h time point. The peak in the number of differentially expressed genes occurred at day 3 (Fig. 3).

Mediators of inflammation comprised the largest functional gene cluster (137 induced and 7 repressed genes). Chemokine and chemokine-related transcripts were among the earliest to change expression, with intercrine and the platelet-derived growth factor inducible proteins JE and KC being significantly induced at the 4 h time point (Fig. 4). The expression of two chemokines, Scya5 and monokine induced by interferon-γ (MIG), were delayed compared with the others, first appearing at 3 days and reaching significant induction at the day 7 time point.

Induction of receptors for a variety of cytokines was detected in the ischemic tissue between days 1 and 10 (Fig. 5 and Supplemental Table 1). Receptors for members of the interleukin family were well represented in the gene list. Receptor mRNA levels increased for interleukins-1, -2, -3, -4, -6, -10, and -17. Receptors for interferons-α, -β, and -γ, tumor necrosis factor, granulocyte-macrophage colony stimulating factor, and granulocyte colony stimulating factor were also induced. Peak expression of these transcripts occurred in most cases between days 1 and 3.

Markers for immune cell infiltration were detected. mRNAs for neutrophil markers peaked at 1 day, followed by those for macrophage and T and B cells (Supplemental Table 1). Complement factor C1q (A, B, and C chains) and the C1q receptor were also induced (Supplemental Table 1).

The largest number of repressed transcripts was found in the functional cluster of genes involved in energy production (Supplemental Table 2). The abundance of mRNAs encoding proteins involved in mitochondrial energy production and fatty acid oxidation were decreased on days 1 and 3, and to a lesser extent day 7 (Fig. 6). During this time period there was a
corresponding increase in mRNA levels for three important enzymes in the glycolytic pathway; α-enolase, phosphoglycerate mutase, and 6-phosphofructokinase. There was also an induction of mitochondrial ATPase inhibitor, a regulatory component of the ATP-synthesizing complex in the mitochondria (Fig. 6).

Another important group of repressed genes were those encoding the adult forms of muscle contractile proteins.
proteins and proteins involved in calcium handling (Fig. 7). Expression for these genes was reduced in the ligated group from days 1–7.

Several markers of cell proliferation were significantly induced above control levels during the week following FAL including: proliferating cell nuclear antigen and cyclin D1 (days 1 and 3); and cyclins A, B1, and B2 and cyclin-dependent kinase 2 (days 3 and 7). The peak in gene expression for each of these factors appeared to be day 3 (Supplemental Table 1). The expression of cyclin D3, which is involved in cell cycle withdrawal, was significantly induced on day 3 and peaked on day 7 (Supplemental Table 1).

Skeletal muscle regeneration is thought in some ways to recapitulate myogenesis, and we detected molecular markers of embryonic muscle development including MyoD and Myf-5 beginning on days 1 and 3, respectively (Fig. 7). We also detected induction of the embryonic forms of myosin heavy and light chains. Real-time, RT-PCR was used to confirm the time course of expression of the myosin adult and fetal isoforms (Fig. 8). The adult isoforms are repressed from days 1 through 7 and return to normal expression levels by day 14, whereas the embryonic isoforms peak at day 7 and return to preligation levels coincidentally with the reemergence of the adult myosins.

The expression levels of several members of the insulin-like growth factor (IGF) axis [IGF-I, IGF-II, IGF binding proteins 3 and 4, growth factor receptor binding protein (Grb10), and IGF binding protein complex acid labile chain] were increased in a time frame consistent with their playing a role in the muscle regenerative process (Fig. 9). The DNA microarray data indicated that there was sequential expression of IGF-I and II as well as IGF binding proteins 3 and 4, and these results were confirmed by real-time, RT-PCR (Fig. 10).

The complete functionally clustered gene list is shown in Supplemental Tables 1 and 2, available online at the Physiological Genomics web site.

DISCUSSION

The results of this study illustrate the remarkable regenerative capacity of the skeletal muscle of young mice to ischemic injury. The muscles most affected by FAL were the soleus and gastrocnemius of the lower leg. There was also evidence of ischemic injury in the...
thigh, but to a lesser extent. During the first 3 days following surgery, there was microscopic evidence of muscle damage and a pronounced loss of function. By day 7, the histological evidence showed a substantial resolution of the injury, and contractile force had been essentially normalized. By 28 days the muscle’s microscopic appearance was largely restored to baseline. Consistent with these findings is the fact that of the 1,289 known transcripts we identified as differentially expressed between ligated and sham surgery controls, none were induced or repressed by the day 28 time point.

Because the gene expression results are too extensive to be discussed in a single paper, we present selective results from the data set that we feel illustrate important elements of the muscle damage and regenerative processes. We also illustrate how this data set may be useful in identifying biological pathways and individual molecules that may be important mediators of muscle regeneration.

Inflammation clearly plays an important role in the response to acute ischemia in this model system. Histological examination showed that immune cell infiltration peaked at 3 days. This correlates with the peak in gene expression; undoubtedly the result of the diversity of cell types entering the ischemic muscle. Transcripts consistent with the appearance of neutrophils, macrophages, and T and B cells were detected, likely recruited by the numerous chemokines that were among the earliest induced genes. Receptor transcripts for members of most of the major families of immune cytokines were also induced.

The infiltrating immune cells provide important factors that not only play a role in the removal of damaged cells, but also in possibly initiating events that lead to muscle repair. Macrophages in particular may be important in stimulating muscle regeneration. Macrophages were shown to be capable of enhancing satellite cell proliferation. They may also help promote myoblast differentiation, but this point is controversial (4, 17).

Two prominent molecular characteristics of the muscle damage caused by ischemic injury are the decrease in expression of transcripts encoding proteins involved in mitochondrial energy production and muscle contraction in the ligated group. The reduced expression of genes in these functional clusters through the first 3 days following ligation is consistent with the diminished contractile force of the hind limb that was observed during this time period.

The regenerative capacity of adult skeletal muscle is dependent upon satellite cells. These cells, which are found in indentations between the sarcolemma and basal lamina (18), are normally in a quiescent or non-proliferative state. In response to muscle injury, satellite cells become activated, proliferate, and fuse to either existing muscle fibers or fuse together to form new muscle fibers (see Ref. 10 for a review of satellite cell physiology and molecular biology). During this process they express myogenic markers, and it is thought that in some ways muscle regeneration recapitulates muscle development.

The regenerative process involves satellite cell proliferation and then differentiation. The histological and functional data give us rough boundaries to these processes, and the molecular markers identified in the gene list help to better define these time courses. The induction of genes associated with cell proliferation, including proliferating cell nuclear antigen, cyclin A, cyclin B1, cyclin B2, cyclin D1 and cyclin-dependent kinase 2, is induced in days 1–7, and all show a peak in expression at day 3 (Supplemental Table 1). Although these are not myocyte-specific signals, the induction of the myogenic basic helix-loop-helix transcription factors MyoD and Myf-5, beginning on day 1 and day 3, respectively (Fig. 7A), are consistent with satellite cell activation and proliferation occurring during this time period. Studies using both primary muscle cells in culture and established muscle cell lines have shown that MyoD and Myf-5 are expressed in proliferating myoblasts prior to differentiation (2). The expression of cyclin D3, which is critical for cell cycle withdrawal, was detected on day 3 and peaked at day 7, and no
cyclin transcripts were induced past the day 7 time point. These results indicate that the process of satellite cell activation and proliferation may begin as early as day 1 and is likely completed within the first week post-FAL.

The expression of myocyte-specific contractile proteins is a marker of myoblast differentiation. We found a peak in the expression levels of the embryonic forms of the myosin heavy and light chains on day 7. The transcript abundance for the adult isoforms of these contractile proteins returned from their nadir to baseline values between days 7 and 14 post-FAL. These results indicate that the process of myoblast differentiation occurs predominately in the second week following acute ischemic injury. Thus, based on transcription profiling, we can roughly determine the time course of the satellite cell activation and proliferation to be occurring from day 1 to day 7 and myoblast differentiation to be occurring between days 7 and 14, with overlap in these processes around the day 7 time point.

Having roughly identified the time course of the two phases of muscle regeneration, it is interesting to pe-
ruse the gene list in search of factors that may, by virtue of their coordinate expression, be possible mediators of these processes. This is illustrated by examining the time course of expression of members of the IGF axis. IGF-I and -II are thought to play an important role in the process of muscle regeneration (for a review of the data see Ref. 14), and our results are supportive of that. The expression of IGF-I and -II have been shown to be transiently induced during myogenesis (1, 5, 8, 9, 13). IGF-I induction is associated with myoblast proliferation (6, 9, 13), whereas IGF-II expression coincides with differentiation (3, 7, 9, 13, 16, 21). We also observed a sequential induction of IGF-I and IGF-II. IGF-I was induced early following FAL (day 1) and showed substantial induction through day 7, the time course when we believe that myoblast proliferation predominates. In contrast, IGF-II expression level did not increase above baseline until the day 7 time point, which is consistent with it playing a role in the process of differentiation. Binding proteins regulate the activity of the IGFs, and we further observed the sequential induction of IGF binding proteins 3 and 4 during the time course of IGF induction. These results are consistent with the hypothesis that these molecules may be working together to mediate the effects of this pleiotropic growth factor axis. Thus, by using coordinate expression as a guide, it may be possible to use this data set to formulate hypotheses regarding biological pathways and individual molecules that play important roles in skeletal muscle injury and regeneration. This may be particularly useful, since most of what is now known concerning these processes is derived from in vitro cell-based assays, and large gaps exist in the signaling molecule, transcriptional regulator, and growth factor axes that mediate adult muscle regeneration in vivo. The fact that the time course of gene expression can also be correlated to histological and functional characteristics of the muscle tissue further enhances its value.

Our data suggest that following ischemic injury skeletal muscle may undergo a transient metabolic and structural adaptation to allow early functional recovery, prior to undergoing complete regeneration. Measurements of muscle function indicate that contractility is substantially restored by day 7. At that time point, however, the adult forms of the myosin heavy and light chains are still at the nadir of their expression profile and the expression of several genes encoding enzymes involved in mitochondrial energy metabolism are also still depressed. The reemergence of the transcripts for the embryonic forms of the contractile proteins, which peak at day 7, and the continued induction of enzymes in the glycolytic pathway at the day 7 time point may help to explain the muscle function data. These results suggest that strategies that promote skeletal muscle functional adaptation by enabling more efficient contraction at low tissue oxygen concentrations may be a useful approach to the treatment of chronic muscle ischemia.

Finally, on a practical note, models of hind limb ischemia induced by FAL have been used to assess the value of exogenous growth factor treatment for the purpose of promoting revascularization (11, 15, 19, 20, 22, 24). The gene expression data presented herein illustrate the complexity of the molecular and cellular response to the ischemic injury. Thus careful consideration should be given to the timing of growth factor addition in light of these results.

As with all large transcriptional profiling studies it is appropriate to discuss the limitations of the results. The method we used to identify differentially expressed transcripts has been previously validated by some of the authors to give a low level of false positives (12). The data in Ref. 12 show that of the 25 genes identified as differentially expressed by DNA microarray analysis at a significance level of $P = 0.05$, 24 of the changes were confirmable to that level of statistical significance using real-time, RT-PCR on the same RNA samples. The $P$ value from TaqMan for the 25th gene was 0.07. Thus we believe that the microarray analysis methodology used in this study provides a reliable list of differentially expressed transcripts, and when we used real-time, RT-PCR to confirm the differential expression of genes identified in this study (Figs. 8 and 10), we found the results to be dependable. Furthermore, many of the genes were found to be induced/repressed at multiple time points and/or by multiple probe sets, which further increases our confidence in the results. However, investigators interested in following-up on results from the gene list are encouraged to independently confirm the differential expression of those genes on a case-by-case basis.

Only positive data from the gene list should be considered meaningful. If a gene is not listed, it does not mean that its expression is not altered during muscle regeneration. There are many reasons why we would not detect differential expression of a particular gene including 1) the probe set needed to identify the target
sequence may not have been on the GeneChip probe array; 2) the probe set for the target sequence may have been on the GeneChip probe array, but the mRNA abundance in the tissue may have been below the sensitivity of the microarray technique; 3) and the precision of the microarrays may not have been sufficient to identify differential expression to the level of statistical significance required.

We made an earnest attempt to cluster the gene list (online data supplement) in a way that would provide the reader with an appreciation for the diversity of pathways that are at work to enable the efficient regeneration of skeletal muscle. Many of the genes fit into more than one functional category, however, and some investigators may reasonably disagree with the assignments.

Finally, we have provided an estimate of the fold induction/repression of the genes based on the microarray analysis in the Supplemental Tables 1 and 2. Although it is our experience that estimates of fold induction/repression from GeneChip probe arrays are generally reliable, it is not always the case. In fact, examination of the Supplemental Tables 1 and 2 will reveal cases where two or more different probe sets that identify the same gene gave widely differing results for levels of induction/repression. Thus it is im-

Fig. 10. Time course of gene expression post-FAL for the members of the IGF axis determined by real-time, RT-PCR. From top to bottom the graphs are: IGF-I; IGF-II; IGF binding protein 3 (IGF BP3); and IGF BP4. The solid circles are the relative mRNA levels for the genes in the FAL group, and the open circles are the values obtained for the sham-operated animals. Values are means ± SE for 6 animals per group. Values are relative to total RNA. *Statistical difference between the two groups (P < 0.05, by unpaired t-test) at that time point.
important for investigators wishing to follow-up on data from Supplemental Tables 1 and 2 to independently confirm the fold change for genes of interest in the model on a case-by-case basis.

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