Mammalian Fetal Cardiac Regeneration After Myocardial Infarction Is Associated With Differential Gene Expression Compared With the Adult

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Background. In adults, myocardial infarction (MI) results in a brisk inflammatory response, myocardium loss, and scar formation. We have recently reported the first mammalian large-animal model of cardiac regeneration after MI in fetal sheep. We hypothesize that the ability of the fetus to regenerate functional myocardium after MI is owing to differential gene expression regulating the response to MI in the fetus compared with the adult.

Methods. Myocardial infarction was created in adult (n = 4) or early gestation fetal (n = 4) sheep. Tissue was harvested after 3 or 30 days, and RNA was extracted for microarray, followed by principal component analysis and global gene expression analysis for the following gene ontology terms: response to wounding, inflammatory response, extracellular matrix, cell cycle, cell migration, cell proliferation, and apoptosis.

Results. Principal component analysis demonstrated that the global gene expression pattern in adult infarcts was distinctly different from the uninfarcted region at 3 days and remained different at 30 days after MI. In contrast, gene expression in the fetal infarct was different from the uninfarcted region at 3 days, but by 30 days it returned to a baseline expression pattern similar to the uninfarcted region. Three days after MI there was an increase in the expression of genes related to all gene ontology terms in fetal and adult infarcts, but this increase was much more pronounced in adults. By 30 days, the fetal gene expression returned to baseline, whereas in the adult it remained significantly elevated.

Conclusions. These data demonstrate that the global gene expression pattern is dramatically different in the fetal regenerative response to MI compared with the adult response and may partly be responsible for the regeneration.

Cardiovascular diseases are the leading cause of death in the United States and worldwide. Every year, more than 1 million Americans experience myocardial infarction (MI) and more than 5 million have heart failure [1]. Although reperfusion and drug therapies greatly contributed to improving the pathophysiology of MI, adverse left ventricular remodeling after MI remains the most common cause of heart failure [2–4]. During the last decade, more interest had been directed to find ways and means to stimulate the regeneration of the infarcted heart. Stem cells constitute a promising strategy to promote myocardial repair and regeneration. However, there are some obstacles that this therapy needs to overcome before it can be applied to patients on a broader scale.

After an MI, a series of events take place starting with an early phase of inflammation characterized by the infiltration of the infarcted area by inflammatory cells [5, 6]. A remodeling stage follows as matrix metalloproteinases and collagenases degrade the extracellular matrix, which eventually results in scar formation, ventricular wall thinning, and a decline in cardiac function [7]. We have recently reported the first mammalian large-animal model of cardiac regeneration after in utero MI in fetal sheep [8]. Using this model, we showed that the fetal response to MI is dramatically different from that of the adult and is characterized by minimal inflammation, lack of fibrosis, restoration of cellularity, increased myocardial proliferation, and restoration of cardiac function [8]. In fact, assessment of fetal and adult hearts after MI demonstrated a decline in adult cardiac function, whereas in the fetus, restoration of cardiac function was observed...
in both ejection fraction and lack of akinetic myocardium. In dramatic contrast to the adult response to infarction, the fetal ejection fraction slightly improved by 3 days, and by 1 month after infarction, the ejection fraction had returned to preinfarction levels. One week after MI, CD45 staining showed high levels of inflammatory cells in the infarct area, which increased even more after 4 weeks, whereas in the fetus, the inflammatory response was minimal [8]. In addition, at 4 weeks there was no obvious apoptosis in the fetal infarcts, whereas the adult infarct showed increased and persistent apoptosis. Moreover, we showed that the regeneration of the myocardium in the fetus is owing partly to the proliferation of differentiated cardiomyocytes and the recruitment of cardiac progenitor cells to the infarct region at 3 days and 4 weeks after MI [9].

In the current study, we aimed to understand the mechanisms of fetal regenerative response to cardiac injury and to identify the factors that regulate the response to wounding, inflammation, and extracellular matrix remodeling. We believe that the expression of specific sets of genes plays an important role in the regulation and control of each of these phases. We hypothesized that the fetal response to MI would be associated with a differential gene expression profile, which may be responsible for the regenerative healing and restoration of myocardial function in the fetal infarct.

Material and Methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of Nemours Children Hospital—Orlando and the University of Pennsylvania and performed in compliance with NIH Publication No. 85-23, revised 1996, and the European Convention on Animal Care.

Myocardial Infarction in Adult and Fetal Sheep

Fetal (65 to 76 days’ gestation, term gestation 140 days) or adult Dorset sheep were used for all studies. Quantitative echocardiography was performed before infarction, immediately after infarction, and at the time of euthanasia. Animals were sedated with ketamine (11 mg/kg intramuscularly), intubated, and anesthetized with inhaled isoflurane. Cefazolin (1 g intravenously) was given before incision, and oxitetracycline (0.06 mg/kg intramuscularly) was administered before extubation for antibiotic prophylaxis.

Myocardial infarction in adult sheep was generated as mentioned previously [10, 11]. For the fetal model, a laparotomy and hysterotomy were performed to expose the fetus. A left thoracotomy was performed, and the pericardium was opened. The left anterior descending coronary artery and appropriate diagonal branches were ligated with sutures to produce an infarct involving 20% of the left ventricular mass. The chest and skin incisions were closed. The amniotic fluid was replaced with sterile normal saline solution with 2 million units of penicillin-G added for antimicrobial prophylaxis. The uterus and abdominal incisions were closed before emergence from anesthesia. Analgesia was provided with buprenorphine (0.005 mg/kg intramuscularly) before extubation, and flunixin meglumine (2.5 mg/kg intramuscularly) was given 4 hours postoperatively. Animals were euthanized at 3 or 30 days after infarction, hearts were excised, and RNA was isolated from the infarct regions of fetal and adult hearts (n = 4 for adult and n = 4 for fetal). The remote regions were isolated as controls.

RNA Isolation and Ovine-Specific Gene Microarray

A 5-mm section of the infarct area or remote region was harvested 3 and 30 days after MI. Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA). Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and then labeled with the incorporation of fluorescent deoxyctydine triphosphate. The mixed probes were then hybridized to the oligonucleotide ovine micorarrays (Agilent Technologies, Foster City, CA). The background-subtracted signal intensities were imported into R statistical environment (www.r-project.org) for statistical analysis, after which lowess normalization of all 15,008 probes was performed across samples. Normalized intensities were further log2 transformed for statistical analyses. Principal component analysis using all probes indicated that the global gene expression patterns were distinguishable between fetal and adult hearts.

Because more than 80% of the probes were mapped to sheep genes not functionally annotated, we mapped microarray probes to human genes through the UniGene database (www.ncbi.nlm.nih.gov/uniGene), which clusters genes across species based on the similarity of protein sequences. There was a total of 9,103 probes mapped to 7,384 unique human Entrez GeneIDs. The difference of group means of each probe was used to represent the differential gene expression between fetal and adult hearts. We then selected several gene groups of interest: response to wounding (GO:0009611; 29 genes), inflammatory response (GO:0006954; 162 genes), extracellular matrix (GO:0031012; 20 genes), cell cycle (GO:0007049; 254 genes), cell migration (GO:0016477; 33 genes), cell proliferation (GO:0008283; 161 genes), and apoptosis (GO:0006915; 235 genes) from the Gene Ontology (GO:0007049; 254 genes), cell migration (GO:0016477; 33 genes), cell proliferation (GO:0008283; 161 genes), and apoptosis (GO:0006915; 235 genes) from the Gene Ontology database (www.geneontology.org), and evaluated whether these genes were differentially expressed as a whole.

Real-Time Quantitative Polymerase Chain Reaction

Heart samples from the remote zone or the infarct were homogenized in TRIZol (Life Technologies, Invitrogen), and total cellular RNA was isolated and purified following the manufacturer’s instructions. For mRNA analysis, mRNA was converted into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time quantitative polymerase chain reaction was performed with the CFX96 real-time polymerase chain reaction thermal cycler (Bio-Rad, Hercules, CA) to amplify samples in triplicate. Relative gene product
amounts were reported for each gene compared with 18S ribosomal RNA. Results were reported as mean ± standard error of the mean.

Statistical Analysis
Student’s t test was used to analyze the data. All data were expressed as the mean ± standard deviation, and a probability value of less than 0.05 was considered to be significant. Table 1 summarizes the Student’s t test probability values for each gene ontology term.

Results
Differential Gene Expression in Infarct Areas From Fetal and Adults Hearts
In this novel model, we generated a unique data set of differentially expressed genes at baseline and after MI in both fetal and adult sheep using the newly available ovine-specific gene expression microarray. Principal component analysis demonstrated that the fetal and adult gene expression patterns in the remote region were different; however, neither remote region varied significantly between 3 and 30 days after infarction (Fig 1). The infarct region in the adult at 3 and 30 days clustered differently and remained distinct from the remote regions at both time points. In contrast, the fetal infarct clustering pattern was distinct from the fetal remote region at 3 days but returned to a similar expression pattern as the remote or uninfarcted fetal myocardium at 30 days after infarction.

Differential Gene Expression of Factors Involved in the Response to Wounding
We analyzed this microarray data set of differentially expressed genes with regard to the gene ontology term response to wounding (GO:0009611). Figure 2 shows the violin plot of this analysis. There was a statistically significant difference in gene expression of factors involved in the response to wounding between the fetus and adult in response to MI. In the adult, the expression of these genes was significantly upregulated in the infarct area compared with the remote zone (uninfarcted myocardium) at 3 and 30 days after MI. Gene expression in the fetal hearts, however, was much lower at 3 and 30 days with the adult heart. The fetal response in the infarct area was higher than in the remote zone at 3 days. After 30 days, this response resolved and came back to the basal level as in the remote zone. Our data demonstrate broad differences in gene expression, in particular to the response to wounding, between the fetal regenerative and the adult reparative response to MI between the remote and infarct areas.

Differential Gene Expression of Factors Involved in the Inflammatory Response
We previously showed that after MI, adult hearts have high and persistent inflammation compared with fetal hearts. So we analyzed this microarray data set with regard to the gene ontology term inflammatory response (GO:0006954). Figure 3A shows the violin plot representation of a statistically significant difference in gene expression of factors involved in inflammatory response between the fetus and the adult in response to MI ($p < 0.001$). As we have seen with the response to wounding, our data from the adult hearts show higher levels of genes involved in inflammation in the infarct area compared with the remote zone at 3 and 30 days. The fetal response, however, was much lower than the adult, and the inflammation was resolved as shown by similar expression levels of inflammatory response genes between the infarct and the remote area at 30 days after MI. Figure 3B shows the real-time polymerase chain reaction analysis of the expression of interleukin 6 (IL-6) and IL-8 in fetal and adult hearts. Both IL-6 and IL-8 are highly expressed in the adult infarct 3 and 30 days after MI. However, in the fetus, 3 days after MI IL-6 gene expression is slightly increased and completely disappears after 30 days.

Differential Gene Expression of Factors That Regulate Extracellular Matrix Remodeling
We recently showed that adult hearts show extensive remodeling of the extracellular matrix, scar formation, and wall thinning at 30 days after MI. We also demonstrated complete myocardial regeneration 30 days after MI. In the current study, we analyzed the microarray data for differentially expressed genes with regard to the gene ontology term extracellular matrix (GO:0031012). Figure 4 shows the violin plot of this analysis with a statistically significant difference in gene expression of factors involved in extracellular matrix remodeling between the fetus and adult at both time points. In contrast, the fetal infarct clustering pattern was distinct from the fetal remote region at 3 days but returned to a similar expression pattern as the remote or uninfarcted fetal myocardium at 30 days after infarction.

Table 1. Number of Genes, Average of (Adult – Fetal), and Probability Values at 3 and 30 Days After Myocardial Infarction

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Number of Genes</th>
<th>Average of (Adult – Fetal)</th>
<th>p Value of Student’s t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to wounding (GO:0009611)</td>
<td>29</td>
<td>0.803</td>
<td>0.013 0.013</td>
</tr>
<tr>
<td>Inflammatory response (GO:0006954)</td>
<td>162</td>
<td>0.343 1.022</td>
<td>0.011 7.8 × 10⁻¹⁰</td>
</tr>
<tr>
<td>Extracellular matrix (GO:0031012)</td>
<td>20</td>
<td>0.180 0.158</td>
<td>0.63 0.7</td>
</tr>
<tr>
<td>Cell cycle (GO:0007049)</td>
<td>254</td>
<td>0.600 0.034</td>
<td>1.3 × 10⁻⁸ 0.55</td>
</tr>
<tr>
<td>Cell proliferation (GO:0008283)</td>
<td>161</td>
<td>0.411 0.284</td>
<td>7.5 × 10⁻⁵ 0.0019</td>
</tr>
<tr>
<td>Cell migration (GO:0016477)</td>
<td>33</td>
<td>0.689 0.884</td>
<td>0.028 0.0032</td>
</tr>
<tr>
<td>Apoptosis (GO:0006915)</td>
<td>235</td>
<td>0.235 0.215</td>
<td>0.0057 0.0071</td>
</tr>
</tbody>
</table>

GO = gene ontology.
fetus and adult in response to MI ($p < 0.001$). The adult hearts showed high expression of extracellular matrix genes 3 and 30 days after MI. In the fetus, on the other hand, we saw much less extracellular matrix remodeling, and after 30 days the expression of genes related to extracellular matrix remodeling returned to normal levels as shown by the violin plots for the infarct and border zones.

### Differential Gene Expression of Factors That Regulate Cell Cycle, Migration, and Proliferation

In Figure 5 we show the violin plot representation of the expression of genes related to cell cycle (GO:0007049), cell migration (GO:0046477), and cell proliferation (GO:0008283). There was a statistically significant difference in the gene expression for all three gene ontology terms between the fetus and adult in response to MI ($p < 0.001$). At day 3 in the adult, our results demonstrate high expression of genes related to cell cycle, proliferation, and migration in the infarct area. At 30 days, however, the expression of the cell cycle and proliferation genes in the infarct was lower than in the remote zone, probably as a result of the formation of an acellular scar as we showed previously [9]. Interestingly, cell migration was much higher in the infarct area at 30 days, which could be owing to the high influx of inflammatory cells into the infarct. In the fetus, on the other hand, gene expression of cell division, proliferation, and migration at 3 days was similar between the infarct and the remote zone but much lower than the adult. At 30 days, all these responses returned to baseline, which could be explained by the complete regeneration of the myocardium at that point.

### Differential Gene Expression of Factors Responsible for Apoptosis

We analyzed the microarray data for the gene ontology term apoptosis (GO:0006915). As shown in Figure 6, infarct samples from adult hearts showed significantly higher expression of genes associated with apoptosis compared with samples from the remote zone. At day 3, fetal infarcts demonstrated higher expression of apoptosis genes compared with the remote zone, but their levels returned to baseline after 30 days.

### Comment

Cardiovascular diseases remain the leading cause of mortality in the United States and worldwide. Every year, more than 1 million Americans are affected by MI, and
despite the currently available therapies, the maximal life expectancy after MI is 5 years [1, 3, 12]. Stem and progenitor cells have shown promising results in promoting myocardial regeneration; however, these therapies are not ready yet to be translated to patients. Unfortunately, these were short-term improvements, and the transplanted cells were unable to engraft in the myocardium and many died after transplantation. One proposed explanation is that the cells are being introduced to an already hostile environment flooded with inflammatory cells, cytokines, apoptotic bodies and mediators, and high levels of reactive oxygen species. This environment would negatively affect the regeneration of the myocardium by limiting the viability of the existent cardiomyocytes and also inhibiting the migration, proliferation, and differentiation of stem and progenitor cells.

In this current study we demonstrate that after MI, fetal and adult hearts present a distinct gene expression profile and marked differences in their response to MI. We show that after MI, fetal hearts present decreased expression of genes involved in the regulation of inflammatory response, extracellular matrix remodeling, apoptosis, cell cycle, cell migration and

Fig 4. Violin plots for the genes related to the gene ontology term extracellular matrix. The y-axis represents the log2 of the ratio of the infarct to remote region average gene expression. The violin shapes represent the distribution of the log2 ratios in each group. The adult infarcts demonstrated increased expression of extracellular matrix genes at day 3 and persistence of the expression at 30 days, whereas the fetal infarct gene expression of extracellular matrix genes returned to baseline by 30 days (20 genes; $p < 0.005$, Student’s t test).

Fig 3. (A) Violin plots for the genes related to the gene ontology term inflammatory response. The y-axis represents the log2 of the ratio of the infarct to remote region average gene expression. The violin shapes represent the distribution of the log2 ratios in each group. The adult infarcts demonstrated increased expression of inflammatory response genes at day 3 and persistence of the expression at 30 days, whereas the fetal infarct gene expression of inflammatory response genes returned to baseline by 30 days (162 genes; $p < 0.005$, Student’s t test). (B) Real-time quantitative polymerase chain reaction analysis of mRNA for interleukin 6 (IL-6) and interleukin 8 (IL-8) in fetal and adult hearts at 3 and 30 days after myocardial infarction. (I = infarct; RM = remote.)
proliferation, and response to wounding compared with adult hearts. We believe that these differences play a crucial role in promoting the cardiac regeneration that we see in the fetal hearts.

Our data show that in the adult hearts, the expression of genes known to play an important role in the response to wounding was significantly upregulated as early as 3 days after MI and remained high even 30 days after injury. In the fetus, on the other hand, the response to injury was not as intense and returned to baseline at 30 days. This distinctive trend in the response to injury between fetal and adult hearts correlates with the echocardiographic data, which demonstrated the healing and complete regeneration of the fetal heart, whereas in the adult, the infarct continued to increase in size and heart function decreased continuously.

From our research in fetal dermal and tendon wound healing, we previously showed that adult dermis or tendon wounds are associated with sustained inflammation and heal with scar formation. Similar wounds in the fetus showed minimal inflammation and healed regeneratively without scar formation. These fetal wounds are characterized by a decreased expression of genes associated with inflammation [13, 14]. The fetal regenerative healing takes place with a diminished inflammatory response, and fibrosis has been associated to inflammation in different organs including the heart [15–18]. In adults, MI induces a dramatic inflammatory response.
leading to scar formation, ventricular remodeling, and decreased cardiac function [5, 7, 19]. We previously showed that there is an association between decreased inflammation and fetal regenerative cardiac healing [8]. In this current study, we show that the global expression of genes closely involved in the regulation of inflammation, apoptosis, and extracellular matrix remodeling in adult hearts is different from fetal hearts after MI. In fact, the reparative healing process in adult hearts correlated with a high and robust expression of inflammatory genes. The fetal heart, in contrast, demonstrated minimal inflammatory response and extracellular remodeling, allowing a regenerative healing to take place.

Injecting stem cells or cardiac progenitor cells into the heart has shown very limited ability to induce cardiac regeneration after MI owing to low number of cells that survive and the inability of most of the injected cells to differentiate into cardiomyocytes. Lafamme and associates [20] used a prosurvival cocktail that inhibited different mechanisms of cell death and improved success levels of embryonic stem cell–derived myocardial graft formation in infarcted rat hearts. These findings support the notion that promoting cardiac progenitor or stem cell proliferation and migration may be a reasonable therapeutic strategy. Our data show that the expression of genes associated with cell cycle and proliferation is significantly higher in adult hearts compared with fetal hearts. This could explain the increased proliferation of cardiac fibroblasts in the adult hearts. Prior studies on fetal hearts showed that the majority of proliferating cells in the fetus are differentiated cardiomyocytes instead of local or circulating progenitor cells [21]. Our data show that in the adult hearts, the signals important for cell migration are dramatically upregulated at days 3 and 30 after MI, but there was no regeneration, which could be attributable to an insufficient amount of stem cells in the adult heart to migrate and repair the infarct. In the fetus, on the other hand, we see a significant decrease in the expression of genes involved in cell migration, especially at 30 days after infarct. This could be explained by the fact that there was a complete regeneration of the infarct at that time, confirmed by echocardiographic data.

In conclusion, this study provides a basis for further research into therapies to minimize apoptosis, avoid fibrosis, promote myocardial regeneration, and maintain cardiac function. We found that there are important differences in the expression of genes associated with the response to injury, inflammatory response, extracellular matrix remodeling, cell proliferation and migration, and apoptosis between the fetal and adult hearts. Understanding the mechanisms behind this differential response could be used to identify and target factors that are crucial to promote a regenerative response and may allow the development of potential treatment strategies to promote cardiac regeneration in the adults.

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