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Regenerative healing following foetal myocardial infarction☆,☆☆

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Abstract

Objectives: The adult response to myocardial infarction results in inflammation, scar formation, left ventricular dilatation, and loss of regional and global function. Regenerative scarless healing has been demonstrated in foetal dermis and tendon and is associated with diminished inflammation. We hypothesised that following foetal myocardial infarction, there would be minimal inflammation, regenerative healing, and preservation of function. **Methods:** Anteroapical myocardial infarction encompassing 20% of the left ventricle was created in adult or early gestation foetal sheep. Myocardial function was serially assessed using quantitative echocardiography. Infarct architecture was examined histologically for evidence of scar formation. Cellular inflammation, cellular proliferation, and apoptosis were assessed using immunohistochemistry. **Results:** In the adult sheep 4 weeks following myocardial infarction, there was a significant decline in ejection fraction (EF) (41 $\pm$ 7.4% to 26 $\pm$ 7.4%, $p < 0.05$), and the akinetic myocardial segment increased in size (6.9 $\pm$ 0.8 cm to 7.9 $\pm$ 1.1 cm, $p < 0.05$). By contrast, there was no decline in the foetal EF (53 $\pm$ 8.1% to 55 $\pm$ 8.8%) and no akinetic foetal myocardial segment 4 weeks post-infarction. The foetal infarcts lacked an inflammatory cell infiltrate and healed with minimal fibrosis, compared with the adults. Foetal infarcts also demonstrated 5-bromo-2′-deoxyuridine (BrdU)+ proliferating cells, including cardiomyocytes, within the infarct. **Conclusions:** These data demonstrate that the foetal response to myocardial infarction is dramatically different from the adult and is characterised by minimal inflammation, lack of fibrosis, myocardial proliferation and restoration of cardiac function. Diminished inflammation is associated with foetal regenerative cardiac healing following injury. Understanding the mechanisms involved in foetal myocardial regeneration may lead to applications to alter the adult response following myocardial infarction.

Keywords: Myocardial infarction; Heart failure; Inflammation; Apoptosis

1. Introduction

The adult response to myocardial infarction (MI) has been well described and follows an orderly sequence of events. It is characterised by an early phase in which inflammatory cells, including neutrophils and T-cells, arrive at the infarct site within the first 72 h [1,2]. Following the inflammatory phase, remodelling occurs within the infarct and is associated with degradation of the extracellular matrix by collagenases and matrix metalloproteinases [3]. The remodelling process of the ventricle following MI in the adult results in ventricular scar formation and is accompanied by ventricular wall thinning, increased wall stress, and a decline in cardiac function [2].

The role of the inflammatory response in post-infarction ventricular remodelling is not fully understood. However, it appears increased inflammation is associated with worse outcomes with neutrophilia being linked to impaired microvascular reperfusion and worsening wall-motion abnormalities following MI [4]. In addition, it has been proposed that the benefit seen with anti-platelet therapy may be partially due to an anti-inflammatory effect targeted against neutrophils [5]. Laboratory studies targeting different components of the inflammatory response following MI have shown improvements in post-infarction left ventricular (LV) remodelling [1,6].

Further evidence to support the role of inflammation in the pathogenesis of the adult response to injury comes from studies in dermal and tendon wound healing. Wounds in adult dermis or tendon are associated with a brisk inflammatory

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cell infiltrate and heal with scar formation, whereas similar foetal dermal or tendon wounds are associated with minimal inflammation and heal by regeneration with a lack of scar formation [7–11]. These foetal wounds have also been shown to have a decrease in the pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) [10,11]. In addition, the deficiency of the anti-inflammatory cytokine interleukin-10 (IL-10) in foetal dermal wounds results in scar formation, and we have recently shown that overexpression of IL-10 in adult dermal wounds decreases inflammatory mediators and inflammation resulting in regenerative or scarless healing [12,13].

To date, the foetal response to cardiac injury is unknown. We hypothesised that the foetal response to myocardial infarction would be associated with minimal inflammation and a lack of scar formation resulting in regenerative healing and restoration of myocardial function, thus preventing the negative sequelae of post-infarction LV remodelling.

2. Materials and methods

2.1. Experimental design

A myocardial infarct model in foetal ($n = 15$) and adult sheep ($n = 23$) was used to investigate ventricular remodelling, the cellular inflammatory response to injury, and cellular proliferation within the infarct over time. Data generated from experimental animals were used for multiple experiments whenever possible to reduce the number of animals needed for the study.

2.1.1. Remodelling experiments

Myocardial infarcts were created in early gestation foetal ($n = 11$) and adult sheep ($n = 19$). The animals were sacrificed at either 3 days (foetal $n = 4$, adult $n = 7$) or 4 weeks (foetal $n = 5$, adult $n = 12$). One set of foetal twins spontaneously aborted prior to sacrifice and was excluded from the study. Echocardiography was performed pre-infarction, post-infarction and just prior to sacrifice to assess the LV function and infarct size. Haematoxylin and eosin (H&E) and Mason's Trichrome staining were used to qualitatively assess the post-MI scar formation and ventricular remodelling. Immunohistochemistry for activated caspase-3 was performed to assess for evidence of apoptosis and cardiomyocyte cell death.

2.1.2. Cellular Inflammatory response experiments

Myocardial infarcts were created in early gestation foetal and adult sheep. The animals were sacrificed at 3 days (foetal $n = 4$, adult $n = 7$), 7 days (foetal $n = 4$, adult $n = 4$) or 4 weeks (foetal $n = 3$, adult $n = 12$), and immunohistochemistry for CD45, the common leucocyte antigen, was performed to assess the level of the cellular inflammatory response in the infarct. The 3-day and 4-week foetal and adult animals were also used in the remodelling experiments.

2.1.3. Foetal myocardial proliferation experiments

Myocardial infarcts were created in foetal sheep ($n = 7$), and 5-bromo-2′-deoxyuridine (BrdU) was given to the pregnant ewe for 48 h prior to sacrifice at either 3 days or 4 weeks. Immunohistochemistry for BrdU was performed to assess for evidence of cell proliferation within the foetal infarcts. The animals used for foetal myocardial proliferation experiments were also used in the remodelling experiments.

2.2. Experimental methods

2.2.1. Animal procedures

All experiments were approved by the Institutional Animal Care and Use Committees of the Children's Hospital of Philadelphia and the University of Pennsylvania and performed in compliance with National Institutes of Health Publication No. 85-23, revised 1996, and the European Convention on Animal Care. Pregnant ewes at 65–76 days gestation or adult sheep were used for all studies. Quantitative echocardiography was performed prior to infarction, immediately after infarction, and at the time of sacrifice. Animals were sedated with ketamine 11 mg kg^{-1} IM, intubated and anesthetised with inhaled isoflurane. Cefazolin 1 g IV was given prior to incision and oxytetracycline 0.06 mg kg^{-1} IM prior to extubation for antibiotic prophylaxis. For the foetal model, a laparotomy and hysterotomy was performed to expose the foetus. In all animals, a left thoracotomy was performed and the pericardium opened to expose the heart. The left anterior descending coronary artery (LAD) and appropriate diagonal branches were suture ligated (Fig. 1(A) and (B)) to produce an infarct involving 20% of the LV mass [14]. The chest and skin incisions were closed. For the foetal cases, the amniotic fluid was replaced with sterile normal saline plus 2 million units of penicillin-G added for antimicrobial prophylaxis; the uterus and abdominal incisions in the ewe were closed prior to emergence from anaesthesia. Analgesia was provided with buprenorphine 0.005 mg kg^{-1} IM prior to extubation and flunixin meglumine 2.5 mg kg^{-1} IM 4 h postoperatively. For

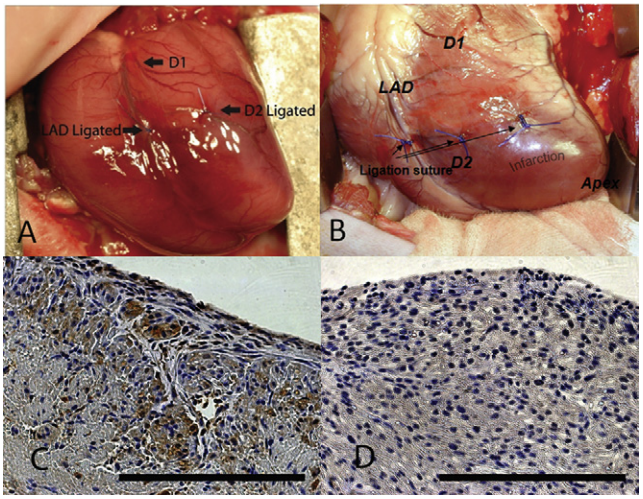

Fig. 1. Ligation of the distal LAD and D2 leads to a reproducible infarct in foetal and adult hearts. An anteroapical MI is created by ligating the LAD and D2 at a point 40% of the distance between the apex and the base in (A) foetal and (B) adult sheep. Activated caspase-3 staining of foetal hearts 3 days following infarction demonstrates (C) apoptosis in the area of infarction (400×) and (D) lack of apoptosis away from the area of infarction (400×). Scale bars equal 200 μm.

the proliferation studies, 48 and 24 h prior to sacrifice, the pregnant ewes received 250 mg of BrdU IV. For the remodelling experiments, animals were anesthetised at 3 days or 4 weeks and had echocardiography prior to sacrifice. The hearts were excised for histological analysis from sheep sacrificed at 3 days, 7 days and 4 weeks.

2.2.2. Echocardiography

Quantitative two-dimensional subdiaphragmatic echocardiograms in the adult and quantitative two-dimensional transuterine echocardiograms in the foetus were obtained before coronary ligation (pre-MI), immediately after coronary ligation (post-MI), and at the time of sacrifice. Echocardiography was performed on a Phillips 7500 (3.5-MHz probe in the adult and 7 MHz in the foetus) and recorded on -in. video tape. Using a modified Simpson's rule technique [15], apical four-chamber views in both foetuses and adults were analysed using an offline analysis system (TomTec Imaging System, GmbH, Unterschleissheim, Germany) to obtain LV volumes at end-systole and end-diastole. The ejection fraction (EF) was calculated from these volumes. Infarct length and LV long axis length were also measured. Infarct size was estimated by the ratio of infarct length to LV length in diastole. This ratio has been found to correlate with quantitative post-mortem assessments of infarct size in this sheep infarct model (unpublished data).

2.2.3. Histology

All tissues were fixed in 10% neutral buffer formalin (NBF) (Sigma–Aldrich) for more than 72 h and processed using a Leica 1050 histoprocessor (Leica Microsystems). Paraffin sections (4 μm) were mounted on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA, USA) and incubated overnight at 52 °F. Slides were deparaffinised in xylene for 10 min × 3 followed by rehydration by graded ethanol (2 × 100%, 95% and 75%) to distilled water. H&E and Masson's Trichrome staining were performed.

2.2.4. Immunohistochemistry

Slides from distilled water were immersed in 0.3% H_2O_2 in distilled water for 30 min at room temperature to quench endogenous peroxidase. They were then rinsed well in distilled water. Antigen unmasking was done using target retrieval (pH 6.0, Dako, Carpinteria, CA, USA) in the microwave (Ted Pella) for 4 min and then cooled to room temperature. Slides were rinsed well in distilled water and serum specific blocking was done with 10% serum for 30 min at room temperature. The serum was tipped off and sections were incubated with primary antibodies overnight at 4 °C. Primary antibodies included CD45 (Serotec, Oxford, UK) mouse anti-ovine at 1:100 dilution, caspase-3 (AbCam, Cambridge, UK) rabbit monoclonal at 1:50 dilution, and BrdU (Invitrogen, Carlsbad, CA, USA) mouse monoclonal at 1:100 dilution. The following day, the primary antibody was tipped off and the slides washed in 0.1 M phosphate-buffered saline (PBS) + Triton. Biotinylated specific secondary antibody, horse anti-mouse or goat anti-rabbit (Vector Lab, Burlingame, CA, USA), was applied at 1:200 dilution for 30 min at room temperature. The secondary antibody was tipped off, and the slides were rinsed in PBS + Triton followed by the ABC complex (avidin–biotin–peroxidase complex, Vector Lab) applied for 30 min at room temperature. After slides were washed in PBS + Triton, the developer with chromogenic substrate (ImmPACT DAB, Vector) was applied for 1–2 min. Slides were then rinsed in distilled water and counterstained with Harris Haematoxylin (Fisher Scientific, Pittsburgh, PA, USA), eosin (Surgipath Medical Industries, Inc., Richmond, IL, USA) or Lichgrün (Croma, Germany). Sections were then dehydrated through graded alcohol series for 2 min each (70%, 95% and 100% × 2), clarified in xylene, and a cover slip applied in Permount (Fisher Scientific, Pittsburgh, PA, USA).

2.2.5. Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA). Non-parametric statistical methods were used for data analysis due to uneven sample size between groups. To examine time-related changes in each of the four different animal groups (foetal 3 day and 4 week, adult 3 day and 4 week), a Friedman's repeated-measures test was used with three time points: pre-MI, post-MI and final MI. When a significant difference ($p < 0.05$) was found within a group, *post hoc* analysis was performed using the Wilcoxon matched-paired test to identify differences between mean values at each of the three time points. Absolute infarct length of the four groups (as listed above) was compared post-MI versus final MI using a Wilcoxon matched-paired test. Post-MI values of IL/DL were compared between foetal and adult sheep using a Mann–Whitney unrelated samples test. Significance was accepted as $p < 0.05$. Values are presented as the mean ± standard deviation.

3. Results

3.1. Ventricular remodelling in the foetus and the adult

Immediately after coronary ligation in both the foetus and the adult, there were easily identifiable wall-motion abnormalities consistent with MI. The ratio of infarct length to LV long axis length in diastole (IL/DL) was 0.92 ± 0.08 ($n = 19$) in the adult sheep and 0.71 ± 0.18 ($n = 9$) in the foetal sheep ($p < 0.05$). In this model, the IL/DL correlates to the infarct size (unpublished data), and this demonstrates that a substantial infarct has been created in both the foetus and the adult when compared to the LV size. Activated caspase-3 staining in foetal infarcts at 3 days confirmed that there was myocyte death within the infarct (Fig. 1(C)).

Qualitatively, all adult sheep had discrete and easily identifiable LV apical aneurysms at 4 weeks (Fig. 2(A)). Apical aneurysms were not present in any of the foetal sheep 4 weeks after MI (Fig. 2(B)).

EF in adult sheep sacrificed at 3 days following MI was 41 ± 9.7% pre-MI, 41 ± 8.2% post-MI, and 34 ± 7.1% 3 days after MI (Fig. 2(D)). There was no significant difference in the EF with respect to time of adult sheep sacrificed 3 days following MI ($p = 0.18$). EF in adult sheep sacrificed at 4 weeks following MI was 42 ± 7.4% pre-MI, 36 ± 6.6% post-MI and 26 ± 7.4% 4 weeks after MI (Fig. 2(D)). EF was shown to be significantly different with respect to time in the adult sheep sacrificed 4 weeks after MI ($p < 0.05$). *Post hoc* analysis demonstrated that there was no significant difference

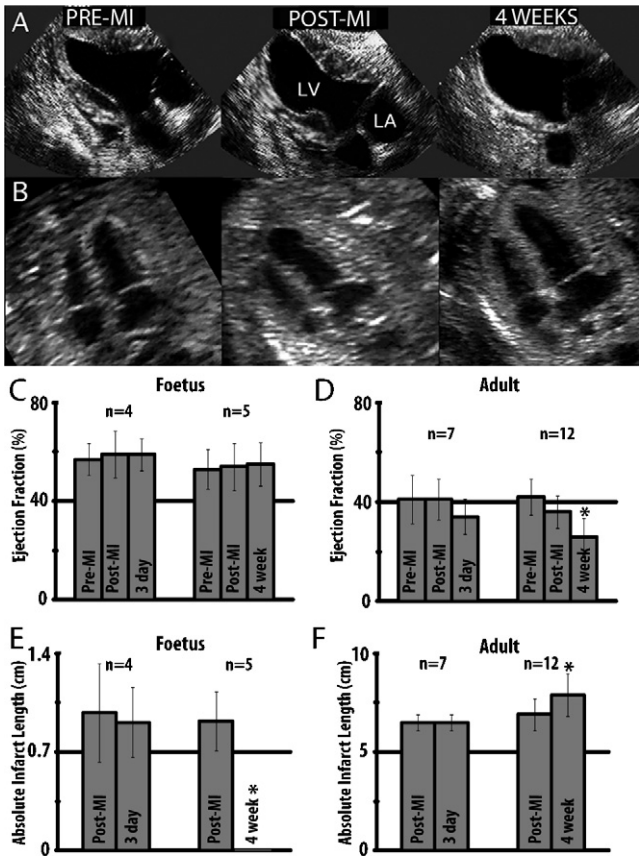

Fig. 2. Echocardioghraphic assessment of foetal and adult hearts following myocardial infarction demonstrates functional decline in the adult and restoration of function in the foetus. (A) Serial end-systolic echocardiographic views demonstrate dilation of the LV, 4 weeks following infarction with a large anteroapical infarct in the adult. (B) In the foetus, there is no evidence of LV dilation or infarcted myocardium at 4 weeks. (C) EF measured by quantitative echocardiography is unchanged in the foetus at 3 days ($p = 0.37$) and 4 weeks ($p = 0.31$) following infarction. (D) In the adult, the EF has significantly declined by 4 weeks following myocardial infarction (*$p < 0.05$ vs adult pre-MI and post-MI). (E) Absolute infarct length defined as the length of akinetic myocardium measured by echocardiography is unchanged at 3 days following infarction ($p = 0.72$) but decreases to zero in the foetus at 4 weeks following infarction (*$p < 0.05$ vs foetal post-MI). (F) In the adult, the absolute infarct length is also unchanged at 3 days following infarction ($p = 1.00$) but increases over a period of 4 weeks following infarction (*$p < 0.05$ vs adult post-MI).

between EF pre-MI and post-MI ($p = 0.10$); however, there was a statistically significant decline in EF at 4 weeks compared with both pre-MI and post-MI ($p < 0.05$). EF in foetal sheep sacrificed at 3 days following MI was $57 \pm 6.6\%$ pre-MI, $59 \pm 9.4\%$ post-MI, and $59 \pm 6.5\%$ 3 days after MI (Fig. 2(C)). EF in foetal sheep sacrificed at 4 weeks following MI was $53 \pm 8.1\%$ pre-MI, $54 \pm 9.6\%$ post-MI, and $55 \pm 8.8\%$ 4 weeks after MI. There was no significant difference in EF with respect to time for foetal sheep sacrificed at 3 days ($p = 0.37$) or 4 weeks ($p = 0.31$) after MI.

Infarct length in the adult sheep sacrificed at 3 days following MI was 6.5 ± 0.4 cm post-MI and 6.5 ± 0.4 cm 3 days after MI ($p = 1.00$) (Fig. 2(F)). Infarct length in the adult sheep sacrificed at 4 weeks following MI significantly increased in size from 6.9 ± 0.8 cm post-MI to 7.9 ± 1.1 cm 4 weeks after MI ($p < 0.05$) demonstrating progressive infarct expansion (stretching) (Fig. 2(F)). Infarct length in foetal sheep sacrificed at 3 days following MI was 0.98 ± 0.35 cm post-MI and 0.91 ± 0.25 cm 3 days following MI ($p = 0.72$) (Fig. 2(E)). Infarct length in foetal sheep sacrificed at 4 weeks following MI was 0.92 ± 0.21 cm post-MI, and no wall-motion abnormality could be detected at 4 weeks following MI ($p < 0.05$) (Fig. 2(E)).

Gross inspection of the adult left ventricle 4 weeks following infarction demonstrates obvious ventricular wall thinning and scar formation in the area of the infarct (Fig. 3(B)), whereas there were no identifiable gross abnormalities seen in the foetal heart (Fig. 3(A)). H&E staining of foetal and adult hearts 4 weeks following MI confirms that there is ventricular wall thinning and myocyte loss in the adult infarcts (Fig. 3(D)), which is not present in the foetal infarcts (Fig. 3(C)). Histological assessment of scar formation using Mason's Trichrome staining demonstrated myocyte loss with extensive fibrosis or scar formation in the adult infarcts (Fig. 3(F)), which corresponds to the akinetic segment of the LV wall seen on echocardiogram. By contrast, the foetal infarcts demonstrated little histological evidence of scar formation 4 weeks following infarction and had myocardium that appeared relatively normal (Fig. 3(E)), which corresponds with the restoration of myocardial function seen by echocardiogram.

3.2. Foetal myocardial regeneration

At 4 weeks, BrdU staining demonstrated isolated areas with disorganised clusters of myocytes in the area of foetal infarction and numerous BrdU-positive cardiomyocytes, consistent with cardiomyocyte proliferation (Fig. 3(G) and (H)). At 3 days following infarction, BrdU staining demonstrated proliferating cells surrounding the area of infarction (Fig. 3(I)) and surrounding blood vessels near the infarct (Fig. 3(J)). These cells were CD45− and therefore likely not of haematopoietic origin (Fig. 3(K)). This is distinctly different from the adult response following MI where there is minimal myocardial proliferation [16]. Myocardial proliferation within the foetal infarct (Fig. 3(G) and (H)) and echocardiographic evidence of fully functional myocardium following infarction (normal EF and no wall-motion abnormality) (Fig. 2(C) and (E)) are consistent with foetal myocardial regeneration.

3.3. Inflammatory response and persistent apoptosis

There was a minimal cellular inflammatory response in the foetal hearts at 3 days, 7 days and 4 weeks following infarction. In the adult sheep, the cellular inflammatory response was greatest at 7 days following infarction, and the adult had numerous CD45+ inflammatory cells in the area of infarction (Fig. 4(B) and (D)) compared with relatively few seen in the foetus (Fig. 4(A) and (C)). At 4 weeks, the adult infarcts continued to have persistent clusters of CD45+ inflammatory cells (Fig. 4(F)) whereas there were minimal inflammatory cells seen in the foetal infarcts (Fig. 4(E)). To assess the level of apoptosis over time following MI, we performed immunohistochemistry for activated caspase-3 staining. Three days following infarction, both foetal and adult infarcts demonstrated apoptosis. However, 4 weeks following MI, adult infarcts demonstrated continued apop-

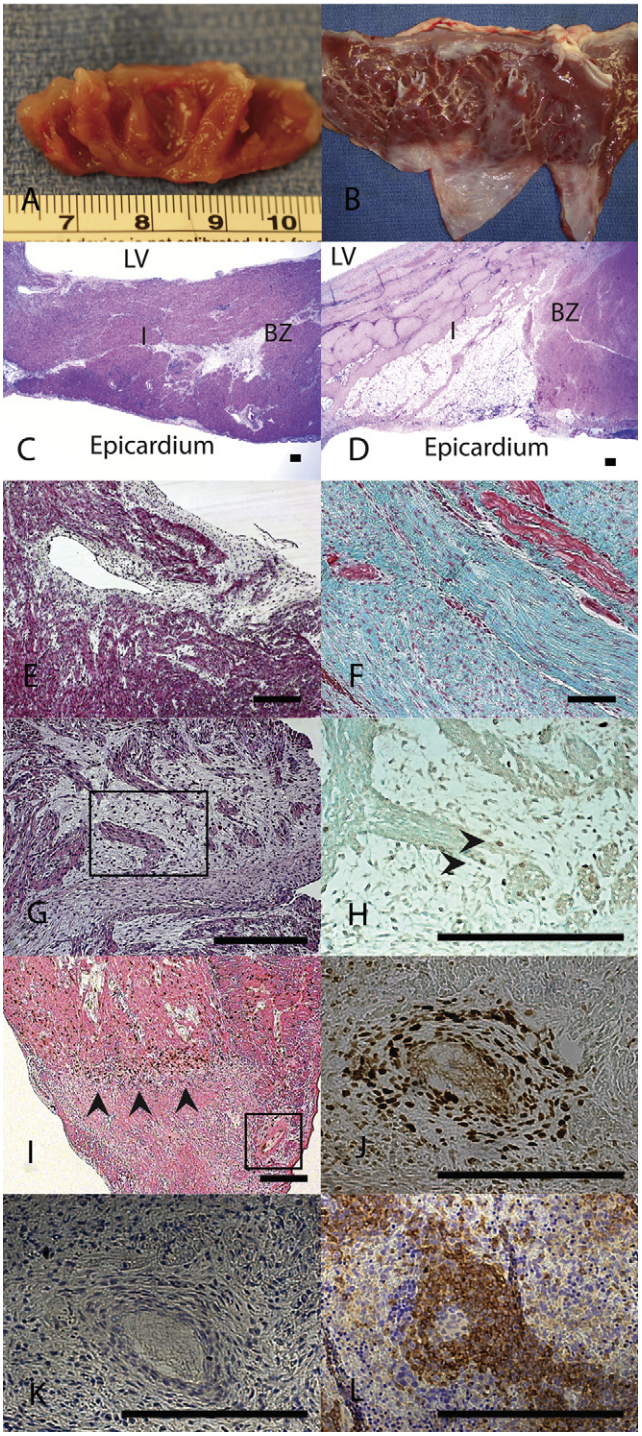

Fig. 3. Foetal cardiac ventricular remodeling following MI proceeds with regeneration of myocardium and without scar formation. 4 weeks after myocardial infarction (A) foetal hearts show no gross evidence of fibrosis while (B) adult hearts show apical fibrosis and ventricular wall thinning. H&E staining at 4 weeks demonstrates (C) no evidence of myocyte loss or ventricular wall thinning in the foetal heart (I) infarct or (BZ) border zone and (D) significant myocyte loss and ventricular wall thinning in the adult infarct (I) (20×). Masson's Trichrome staining at 4 weeks following MI confirms that there is (E) minimal fibrosis in the foetal infarct (100×) and (F) an exuberant fibrotic response in the adult infarct (100×). (G) 4 weeks following myocardial infarction, H&E staining of the foetal infarct shows disordered clusters of cells with minimal surrounding fibrosis suggesting myocardial regeneration (200×). (H) BrdU immunostaining with Lichgrün counterstain on a serial section demonstrates that within these clusters, there are cycling, BrdU positive, cardiomyocytes marked by the arrowheads confirming myocardial proliferation within the infarct (400×). (I) BrdU immunostaining with eosin counterstain on a foetal heart 3 days following MI demonstrates a polarity for BrdU-positive cells which accumulate around the area of infarction (marked with arrowheads, 50× optical, 2× digital) and (J) surrounding blood vessels within the infarct (serial section with Lichgrün counterstain, 400×). (K) CD45 immunostaining on a serial section of this blood vessel within the foetal infarct demonstrates that these BrdU-positive cells are not positive for CD45, indicating they are not of haematopoietic lineage. (L) Positive control of CD45 immunohistochemistry on a foetal sheep spleen. Scale bars equal 200 μm.

tosis (Fig. 4(H)). By contrast, there was no observable activated caspase-3 staining in the foetal infarct 4 weeks after MI (Fig. 4(G)).

4. Discussion

Our findings demonstrate a marked difference between the foetal and adult responses to myocardial infarction. The adult paradigm of healing by reparative scar formation was demonstrated by the replacement of myocardium with collagen, infarct expansion and decline in cardiac function and was associated with a robust and prolonged cellular inflammatory response. The foetus, on the other hand, demonstrated the capability of healing by myocardial regeneration and was associated with minimal cellular inflammatory response and cellular proliferation both in the foetal infarct and border zone. This provides the first model of regenerative healing in the heart following MI and demonstrates an association between the cellular inflammatory response, infarct expansion and ventricular remodelling following injury.

Acute MI afflicts over 1 million Americans each year, and despite modern reperfusion and pharmacologic therapy, the 5-year post-MI mortality remains near 20% with 70% of all heart failure cases due to post-MI LV remodelling [17–19]. Therefore, loss of myocardial function following MI is the cause of significant morbidity and mortality, and the foetal response to MI demonstrates that complete restoration of cardiac function with no pathologic LV remodelling is possible. We believe that this novel model of regenerative cardiac healing can be used to identify factors that are important in promoting the regenerative phenotype as has been done in other tissues. Based on our research in foetal scarless dermal healing, we have developed strategies to alter the wound environment and promote regenerative healing in the adult [12,13]. The same approach should be applied to investigate novel therapies to promote cardiac regeneration and minimise the pathologic LV remodelling that is seen in the adult.

Our model of foetal regenerative cardiac healing uses a permanent coronary artery ligation that was developed from an adult ovine MI model that has been shown to result in significant pathologic LV remodelling and decline in function [14]. The foetal and adult models both used ligation of the LAD and D2 at points 40% of the distance from the apex to the base. In the adult, we have previously correlated the size of the infarct with the IL/DL ratio. In this study, we found that although we used an identical ligation strategy in the foetus

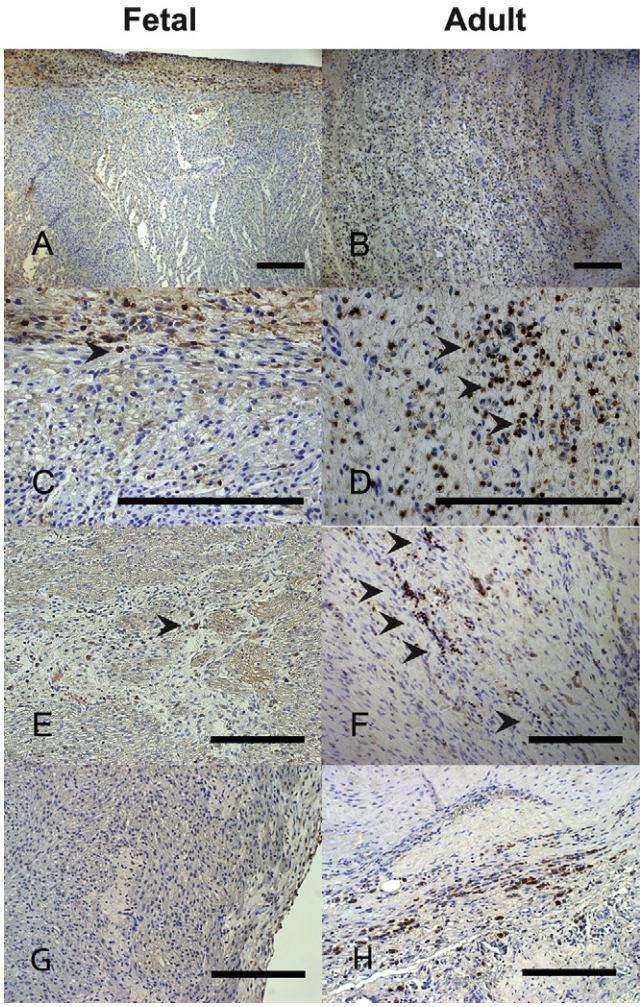

Fig. 4. CD45 immunmohistochemistry following myocardial infarction demonstrates markedly less cellular inflammatory response in foetal verses adult hearts. 7 days following infarction the foetal heart (A: 100×, C: 400×) shows minimal numbers of inflammatory cells while the adult heart (B: 100×, D: 400×) shows a large inflammatory infiltrate. At 4 weeks following infarction, the number of inflammatory cells in both the (E) foetal and (F) adult hearts has decreased, but the adult heart has persistent scattered areas of inflammation not seen in the foetus (200×). (G) Caspase-3 staining in the foetal heart 4 weeks after infarction fails to show any apoptosis (200×). (H) Caspase-3 staining in the adult infarct 4 weeks after infarction demonstrates continuing apoptosis (200×). Scale bars equal 200 μm.

and the adult, the IL/DL ratio is slightly smaller in the foetus. This difference may be the result of small differences in echocardiographic technique between adult and foetal animals. While it is possible that these data indicate that the foetal infarcts encompass a smaller portion of the LV mass, the consistency of our surgical technique as well as echocardiographic and direct visual inspection of a large wall-motion abnormality strongly suggest that foetal hearts were subjected to a large infarction and a strong remodelling stimulus.

Our data demonstrate an association between decreased inflammation and foetal regenerative cardiac healing. Studies in other tissues have also demonstrated that foetal regenerative healing proceeds in the face of a diminished inflammatory response [7–11]. Myocardial infarction in the adult has been shown to induce a dramatic inflammatory response, resulting in ventricular scar formation and leading to progressive infarct expansion and LV aneurysm formation [2,4,14]. The adult infarcts in this study also demonstrated a prolonged inflammatory cell infiltration and ongoing apoptosis. While our study does not prove that increased inflammation is causally linked to pathologic LV remodelling and continued cardiomyocyte cell death, it is reasonable to hypothesise that this is the case. Inflammation has been linked to fibrosis in multiple different organs including the heart [20–23]. Given the strong association we have shown between foetal regenerative cardiac healing and diminished inflammation, it is possible that cellular inflammation following MI in the adult affects downstream targets driving fibroplasia and potentiating ongoing cardiomyocyte apoptosis. However, further studies are needed to demonstrate a causal relationship. This study provides a basis for further research into therapies suppressing the inflammatory response following MI with the goal of minimising ongoing apoptosis, promoting myocardial regeneration, preventing fibrosis and preserving function.

While the inflammatory response may be one key difference between foetal regenerative cardiac healing and adult reparative cardiac healing, there are many other potential differences, which may contribute to regenerative healing in the foetus and pathologic LV remodelling in the adult. We hypothesise that there are important differences in the extracellular matrix, cell migration, gene expression and progenitor cell function between the foetal and adult hearts. Future studies are needed to identify additional factors that control foetal regenerative cardiac healing and adult pathologic LV remodelling following MI.

Our data clearly demonstrate regeneration of myocardium following MI in the foetus, but it is not clear what cells are most important in this process. Our findings suggest that proliferation of differentiated cardiomyocytes is partially responsible for the regenerated myocardium. Prior studies on foetal cardiac regeneration support this finding and have demonstrated that the majority of proliferating cells in the foetus are differentiated cardiomyocytes instead of either local or circulating progenitor cells [24]. The increased cellular proliferation that we observed surrounding the infarct 3 days after foetal MI is unlikely exclusively due cardiomyocyte proliferation in the border zone. A portion of the cellular proliferation observed is likely due to progenitor cell populations and endothelial cells. Further studies are needed to better define the contributions of these cell populations to foetal myocardial regeneration following MI.

One caution when interpreting our study is that comparing foetal cellular processes with those of the adult may have a confounding variable of the stage of development. At this time, there is no model of foetal pathologic LV remodelling following MI. Development of such a model would eliminate this confounding variable; however, using the models described in our study to compare foetal regenerative cardiac healing with adult reparative cardiac healing still has the potential to identify important factors controlling cardiac regeneration following MI. Studies comparing foetal scarless wound healing with adult scar formation have identified important factors, such as IL-10, that can be used therapeutically to alter the adult healing response [13]. We propose that using this model of foetal regenerative cardiac

healing in future research has the potential to further our knowledge of cardiac regeneration and lead to novel strategies to alter the adult response to MI. However, future investigations should also take into consideration the effect that stage of development has on cellular processes.

In conclusion, there has been little change in the natural history of post-MI ventricular remodelling and the associated morbidity and mortality over the past 50 years despite robust research efforts on the topic [25]. It is clear that following a large LV myocardial infarction, replacement of myocardium with collagen scar does not preserve function over time. Our research demonstrates that diminished inflammation is associated with foetal cardiac regenerative healing, and we propose that intensive research into other factors promoting foetal regenerative healing following MI will allow for the development of therapies that can promote regenerative healing in the adult.

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Appendix A. Conference discussion

Dr H. Klein (*Duesseldorf, Germany*): Unlike some papers from the early '90s that perhaps raised more questions than provided answers, maybe you are able to offer some elucidation.

Do you think the main issue here is that of inflammation as part of the contact activating system, or, rather, this being some sort of prevention? Or what other mechanisms do you suggest to be potentially responsible for the myocardial regeneration?

Dr Herdrich: Well, I think that the regenerative cardiac healing seen in the foetus is multifactorial in nature. I think that inflammation likely plays a very important role.

If you look at studies that were done in other tissues, dermis, tendon in particular, inflammation seems to be an important piece in controlling whether or not something will heal by regeneration or by scar. And there are studies that have utilised what has been identified in, for instance, foetal scarless wound repair, and transferred that to adult models and shown regenerative healing in adult animal models.

So I think inflammation is very important; however, we have a long way to go in fully understanding what aspects of the inflammatory response are key and how we can use those to alter the adult response.

Dr Klein: So your observations once again indicate immune modulation, and anti-inflammation may be the primary beneficial mechanism of cellular cardiomyoplasty. Did you check in your work toll-like receptors or other surface markers?

Dr Herdrich: No, we did not. This is the beginning of a very large project, and that's certainly something that could be done in the future. We have lots of plans, and that could be one of them, but we have not done that to date.

Dr Klein: In the context of doing cell therapy, we know today that there is virtually no trans-differentiation or cell fusion, so we do not really generate new cardiomyocytes. What might rather be the case is that we initiate a re-remodelling of the connecting tissue and the extracellular matrix.

When comparing foetal and adult sheep, did you find there to be any difference in expressing mediators or surface markers with reference to the matrix?

Dr Herdrich: Well, part of what we are currently doing is looking at gene expression within both of these models. We don't have that data available yet, but it's certainly part of our ongoing investigation.

I will say with regards to your comments regarding prevention and remodelling, that I think it's fairly clear in that we've shown that in our foetal infarct model, we get a true infarct. We have a sustained echocardiographic evidence of a sizable infarct in the foetus that goes at least through day 3 and then resolves by 4 weeks. And so we do get an infarct, and we've coupled that with immunohistochemistry showing apoptosis in the foetal hearts.

On the other side in the adult, you have continued apoptosis throughout the study period, so you have continued myocardial cell death, and you don't see that in the foetus.

Dr Klein: But on the other hand, you know that in adults we might need to trigger the homing effect, especially in chronic stages. That is those patients being treated by the cardiac surgeons for chronic heart failure.

If we initiate any kind of inflammation, for instance physically by using light energy or shock waves, this will potentially instigate an additional benefit of cell therapy. Can you comment on any experience you might have had in this regard with your work?

Dr E. da Cruz (*Denver, Colorado, USA*): I'm very sorry to interrupt, and this is fascinating, but, unfortunately, for the sake of time, we must proceed. I'm sorry. We can stay here for hours and hours discussing this.