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Cardiomyocyte Enlargement, Proliferation and Maturation During Chronic Fetal Anemia in Sheep

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Abstract

Objectives—Chronic anemia increases the workload of the growing fetal heart, leading to cardiac enlargement. To determine which cellular process increases cardiac mass we measured cardiomyocyte sizes, binucleation as an index of terminal differentiation, and tissue volume fractions in hearts from control and anemic fetal sheep.

Methods—Fourteen chronically catheterized fetal sheep at 129 days gestation had blood withdrawn for nine days to cause severe anemia; 14 control fetuses were of similar age. At postmortem, hearts were either enzymatically dissociated or fixed for morphometric analysis.

Results—Daily isovolumetric hemorrhage reduced fetal hematocrit from a baseline value of 35% to 15% on the final day ($P < 0.001$). At the study conclusion, anemic fetuses had lower arterial pressures than control fetuses ($P < 0.05$). Heart weights were increased by 39% in anemic fetuses compared to control hearts ($P < 0.0001$), although the groups had similar body weights; the heart weight difference was not due to increased ventricular wall water content or disproportionate non-myocyte tissue expansion. Cardiomyocytes from anemic fetuses tended to be larger than those of control fetuses. There were no statistically significant differences between groups in the cardiomyocyte cell cycle activity. The degree of terminal differentiation was greater in the right ventricle of anemic fetuses compared to controls by ~8% ($P < 0.05$).

Conclusions—Anemia substantially increased heart weight in fetal sheep. The volume proportions of connective and vascular tissue were unchanged. Cardiomyocyte mass expanded by a balanced combination of cellular enlargement, increased terminal differentiation, and accelerated proliferation.

Keywords

heart; hyperplasia; terminal differentiation

The fetus responds to chronic anemia by increasing its cardiac output by ~50% over normal levels, accelerating heart rate, and boosting biventricular stroke volume (Davis and

Hohimer, 1991). These responses augment the work of the growing heart, leading to cardiac enlargement and expansion of the coronary tree (Oberhoffer *et al.*, 1999; Davis and Hohimer, 1991; Davis *et al.*, 1999; Olson *et al.*, 2006). Growth responses of the fetal heart to altered hemodynamic load are complex and poorly understood. While increased systolic blood pressure is known to stimulate cardiac growth in fetal sheep (Barbera *et al.*, 2000; Jonker *et al.*, 2007a), the accelerated cardiac growth of anemic fetuses occurs despite a decrease in mean arterial pressure (Davis and Hohimer, 1991).

Growth of the fetal heart occurs normally by cardiomyocyte proliferation and enlargement (Jonker *et al.*, 2007b), with proportional growth of the non-myocyte cardiac tissues (Smolich *et al.*, 1989). However, the cellular changes that lead to cardiac growth in the near-term fetus are specific to the nature of the stimulus. For example, ventricular systolic hypertension imposed by a pulmonary artery occluder causes cardiomyocyte enlargement and early terminal differentiation, as well as a reduction in the proportion of non-myocyte tissues in the myocardium (Barbera *et al.*, 2000). In contrast, elevation of cortisol to sub-pressor levels in fetal sheep causes cardiac enlargement by cardiomyocyte proliferation (Giraud *et al.*, 2006). Abnormal stimuli thus typically alter, rather than simply accelerate, the normal course of cardiomyocyte growth and maturation patterns in the fetal sheep.

Several aspects of cardiac anatomy and physiology are determined by changes in the fetal environment and the timing of maturational events. For example, sheep that were transiently anemic *in utero* have almost twice normal maximal coronary conductance in adulthood (Davis *et al.*, 2003). Unlike working myocytes in the adult heart, fetal cardiac myocytes can proliferate throughout most of gestation (Jonker *et al.*, 2007b). Fetal proliferative growth ceases around the time of birth when cardiomyocyte terminal differentiation occurs; this maturational event suggests that maximum cardiomyocyte number may be set in this period. Thus, while we know that the coronary vasculature is greatly altered for life in the anemic fetus, we know little about the cellular growth patterns of the fetal heart as it responds to the host of physiological changes that accompany moderate to severe anemia. We do not know in the chronically anemic fetus whether cardiac mass is increased by cardiomyocyte hypertrophy, proliferation, or by expansion of the non-myocyte tissue fraction, or if terminal differentiation is accelerated.

Because arterial pressure, a stimulus for cardiomyocyte enlargement, tends to be lower than normal in chronically anemic fetuses, we hypothesized that the type of hemodynamic volume load imposed by chronic fetal anemia would increase cardiac mass by causing cardiomyocyte proliferation, while reducing the proportion of non-myocyte myocardial tissue.

Methods

Ethical Approval

All animal protocols were conducted under the auspices of the Institutional Animal Care and Use Committee and the veterinary staff of the Department of Comparative Medicine, according to the *Guide for the Care and Use of Laboratory Animals*.

Animal Preparation

Time-bred ewes of mixed western breed were obtained from a local supplier and acclimatized to the laboratory. Fasted ewes with fetuses aged 122 days of gestation (dGA, term is ~145dGA) were given atropine (7.5mg i.m.) prior to induction of anesthesia with diazepam (10mg i.v.) and ketamine (400mg i.v.). A surgical level of anesthesia was maintained by ventilation with isoflurane (1.5%) in a 70:30 mixture of oxygen and nitrous oxide. Sterile surgical technique was used to expose the uterus through a mid-line incision.

The fetal head was then exteriorized and catheters placed into a fetal carotid artery and jugular vein (0.86mm or 1.19mm internal diameter; Scientific Commodities Incorporated, Lake Havasu City, AZ). An amniotic fluid catheter (1.19mm internal diameter) with multiple side-openings was anchored to the fetal skin. Following closure of the uterus, 1 million units of penicillin G were infused into the amniotic fluid. Incisions were closed in layers, and catheters were tunneled subcutaneously to emerge on the ewe's flank. Ewes received buprenorphine (0.6mg i.m.) twice daily for 2 days as routine postoperative pain medication. Animals were allowed to recover for about 7 days prior to beginning the experimental protocol. Sixteen ewes underwent surgery to instrument 27 fetuses, of which 4 were singletons and the remainders were twins. Thus there was a total in the anemia group of 14 fetuses, and in the control group 13 catheterized fetuses as well as 1 uninstrumented fetus. Each non-anemic fetus served as a control for its twin; normal singletons were designated as paired controls to anemic singletons.

Experimental Protocol

Intravascular pressures were measured continuously with the ewe in a stanchion where she was afforded free access to food and water and freedom to stand or lie down. Pressures were recorded using Transpac pressure transducers (Abbott, Abbott Park, IL) on a calibrated computerized system (ADInstruments, Colorado Springs, CO; Apple, Cupertino, CA). Fetal intravascular pressures, reported as arithmetic means calculated from computer tracings, were referred to amniotic fluid pressure. Heart rates were calculated from arterial pressure tracings. Daily fetal arterial blood samples were taken for determination of blood gases, pH and hematocrit. Fetuses randomly allocated to the anemia group had blood removed according to a formula determined empirically during previous studies in the laboratory to rapidly induce anemia with minimal acute fetal demise (success rate =76%). The target daily bleed volume was 100ml (~30% total blood volume) until the oxygen content was below 4ml/dl, and thereafter the target bleed volume was $44 \times [\text{oxygen content}] - 75\text{ml}$. In the anemic group, an average of 78ml blood was replaced with saline daily for the first 5 experimental days, and an average of 37ml of blood was replaced daily during the remainder of the experiment. Experiments were conducted for 9 ± 1 days; three fetal experiments were concluded when the ewes (each carrying one anemic and one control fetus) went into labor. Experiments were concluded when the fetuses were $137 \pm 1\text{dGA}$.

Postmortem

At the conclusion of the experimental period, ewes were euthanized with a commercially available preparation of sodium pentobarbital (65mg/kg i.v.). Deeply anesthetized fetuses were given heparin (5000U i.v.) and saturated potassium chloride (10ml i.v.) to arrest their hearts in diastole. At post-mortem, fetuses and their excised hearts were weighed. The hearts were assigned to either a dissociation protocol to study isolated myocytes or a fixation protocol to study cardiac morphology and histology.

Cardiac dissociation and cardiomyocyte analysis

Prior to cardiac dissociation in 3 control and 3 anemic fetuses, a full-thickness meridional section of left ventricular (LV) freewall superior to the apex was excised and weighed. These sections were oven-dried until of constant weight, and were used to determine wet/dry myocardial weight. Fetal hearts were enzymatically dissociated, cardiomyocytes were fixed, and myocyte measurement and analysis was performed as has been previously described in detail (Jonker *et al.*, 2007b). Cardiomyocytes so prepared are readily distinguishable from other cardiac cells due to their torpedo shape and regular striations. Cardiomyocyte lengths and widths were measured in 100 myocytes from each ventricle separately for each fetus. As has been previously described, a shape constant or correction factor to calculate myocytes volume was determined from mononucleated and binucleated myocytes ($n \approx 10$ each) from

both ventricles of 5 control and 5 anemic fetuses. In short, it was assumed that myocytes were symmetrical around their long axis and that the shape of each myocyte could be approximated by a series of truncated right circular cones. A shape constant was then derived from the ratio of this measurement to the volume of a cylinder described by the myocyte's length and width. Binucleation was determined by counting the number of mono- and binucleated cardiomyocytes in a sample of 300 cells. The MIB-1 antibody (DAKO, Carpinteria, CA) against the Ki67 antigen was used to immuno-stain for cell cycle activity; no fewer than 500 myocytes from each ventricle were counted separately for each fetus. Positively-stained myocytes were scored according to criteria described previously, and are expressed as a percentage of the mononucleated cardiomyocytes.

Cardiac fixation and morphometric analysis

Hearts to be fixed were perfused for 5 min at 35mmHg pressure via the coronary arteries (which is associated with no apparent edema) with phosphate-buffered saline (PBS) containing 1% purified bovine serum albumin and 1% adenosine to dilate the coronary arteries. Ventricular distension pressure was approximately 3–4mmHg during coronary perfusion. Hearts were perfused with freshly made fixative solution (2% formaldehyde in PBS, pH 7.4) for 20 min and stored in the fixative overnight. Hearts were sliced into 5mm transverse blocks from the level of the atrioventricular valves to the apex. A digital image of the apex and base sides of each block and a centimeter ruler was made using a flatbed scanner (Canon, Lake Success, NY). Scanned ventricular images were processed in NIH ImageJ using the image of the centimeter ruler to calibrate each image (Abramoff *et al.*, 2004; Rasband, 2006). *Radius of curvature* – To determine radius of curvature, a best-fit circle was found to best describe the arc of the ventricular freewall from the block mid-way between the apex and base. Measurements from the epicardial and endocardial surfaces of both the base and apex-side block images were averaged to determine mid-ventricular freewall radius of curvature. *Wall thickness* – A straight line measurement was taken of freewall thickness in the area most remote from the septal attachment. Measurements from both the base and apex-side block images were averaged to determine freewall thickness. *Wall stress* – Ventricular radius of curvature (r), freewall thickness (h), and the mean arterial pressure on the final experimental day (p) were used to estimate wall stress (S_w) according to the Laplacian relationship:

$$S_w = pr/2h$$

Ventricular Volume

To determine ventricular freewall volume, the area of each slice was multiplied by the height of the slice. Area measurements traced from the basal and apical surfaces of each slice were averaged before calculating volume.

Volume fractions

Myocardial blocks were embedded in paraffin and sliced into 5µm sections. These sections were stained with Masson's Trichrome. Stained sections were visualized at 40× magnification, and 5 photomicrographs were taken of randomly selected locations from each ventricle. Volume fraction of specific structures was calculated from an unbiased estimate based on random point counting techniques as described previously in this laboratory (Barbera *et al.*, 2000). A grid was placed over each tissue photomicrograph such that the points fell randomly on specific structures, and each point was categorized as an item to be counted. Each point was assigned a category or, if ambiguous, as one-half point in each of the appropriate categories. Cell and non-cell volume fractions were determined.

Myocyte calculations

The number of myocytes was calculated as described previously (Jonker *et al.*, 2007b) by dividing the total weight of the ventricular freewall in grams (corrected for the volume fraction that is myocyte) by the volumes of the cardiomyocytes in milliliters, taking into account the fractions and volumes of mononucleated and multinucleated cells for the LV and RV.

Plasma assays

Radioimmunoassays were used to measure plasma cortisol (Diagnostic Products, Los Angeles, CA) levels. Protein concentrations were measured using the bicinchoninic acid-based assay from Pierce (Rockford, IL).

Statistical analysis

Fetal blood gases, hematocrits and intravascular pressures were compared using repeated measures one-way analysis of variance (ANOVA). Where justified by the F statistic, Bonferroni's post-test was used to compare baseline and final day within, and between, groups. Weights, cardiac morphometry, histology, and cardiomyocyte measurements were compared (anemic to control, within ventricles) using paired t-tests. A sign test was used to determine the probability that anemic fetus myocytes dimensions were similarly different from control animal myocytes dimensions. All statistics were performed using Graphpad Prism version 4.0a for Mac OS X (Graphpad Software, San Diego, CA). A probability value (P) less than 0.05 was accepted as significant. Data in text and tables are presented as mean $\pm$ standard deviation (SD).

Results

Fourteen fetal sheep were hemorrhaged daily for 9 ± 1 days and compared to 13 similarly instrumented fetuses and one uninstrumented fetus over the same time period. Daily isovolumetric hemorrhage reduced fetal oxygen content from a baseline value of 8.1 ml/100ml to 3.3 ml/100ml on day 4 (Figure 1), and was thereafter maintained at a low value in experimental fetuses. On the final day the mean oxygen content of anemic animals was 2.3 ml/100ml, statistically significantly lower than the anemic baseline and age-matched control values (Table 1). Hemorrhage did not affect arterial PCO₂, but fetal pH was reduced on the final day in the experimental group compared to baseline. Hematocrit was reduced by daily hemorrhage from 35% at baseline to 15% on the final day. Arterial PO₂ was 17mmHg in the anemic group at the conclusion of the experiment, slightly reduced from the baseline and final day control values of 20mmHg. Consequently, the drop in oxygen content of arterial blood in the hemorrhaged group occurred primarily due to reduced hematocrit, but also in small part to a decreased PO₂.

Anemic animals did not undergo the normal slight age-related increase in arterial pressure over the course of the experiment. Thus, mean arterial pressure was reduced slightly in the hemorrhaged group compared to the control group on the final day (Table 1, $P < 0.05$). A trend for the typical age-related decline in heart rate was found in the control fetuses, but not the anemic fetuses (no statistically significant difference by post-test). Specific differences in measurements of right atrial pressures were also not found between groups.

Plasma cortisol levels were not different between anemic and control fetuses (Table 1). A large increase, normal near term, was observed between baseline and final day in both groups.

After 9 days, heart weight was increased by 39% in anemic fetuses compared to controls, although the groups had similar body weights (Table 2). Thus the heart to body weight ratio was increased following prolonged fetal anemia. The LV wet-to-dry weight ratio was not different between the groups.

The gross morphology of the fetal hearts changed with hemorrhage, enlarging while preserving mid-ventricular wall stress (Table 3). The LV mid-wall radius of curvature was greater in anemic fetuses than control fetuses by 11% ($P<0.05$), although this measure was not statistically significantly different in the right ventricle (RV). Likewise, RV myocardial mid-wall thickness was increased by 16% in anemic fetuses ($P<0.05$). These results are true with and without normalization for fetal body weight.

RV binucleated myocyte length was the only individual measurement to be significantly larger in anemic fetuses compared to controls ($P<0.05$). However, all linear cardiomyocyte dimensions tended to be larger in anemic fetuses than control fetuses (Table 3); the probability that all 8 of these measures would be different in the same direction due to chance is $P=0.008$. The shape constants (to calculate myocyte volume from length and width measurements) for mononucleated myocytes were not different between groups or ventricles (control LV: 0.56 ± 0.09 , RV: 0.58 ± 0.06 ; anemic LV: 0.55 ± 0.06 , RV: 0.58 ± 0.04). Similarly, the shape constants for binucleated myocytes were not different between groups or ventricles (control LV = 0.56 ± 0.04 , RV = 0.61 ± 0.06 ; anemic LV = 0.56 ± 0.04 , RV = 0.58 ± 0.06).

The proportion of the cardiomyocytes that were binucleated was greater in the RV of anemic fetuses compared to controls (Figure 2A ; control $49\% \pm 11\%$; anemia $56\% \pm 9\%$, $P<0.05$). This difference was not statistically significant in fetal LV cardiomyocytes (control $49\% \pm 13\%$; anemia $56\% \pm 7\%$). Cell cycle activity, as a percent of mononucleated cardiomyocytes, was not affected by anemia (control LV: $4.2\% \pm 2.4\%$, RV: $7.3\% \pm 4.0\%$; anemia LV: $4.5\% \pm 2.0\%$, RV: $6.4\% \pm 2.2\%$; Figure 2B).

In the ventricular freewalls of near-term fetal sheep, cardiomyocyte cell volume accounted for approximately 80% of myocardial volume (Table 4). Blood vessels (lumen and wall) occupied approximately 14%, while the remaining 6% was composed of connective tissues. There were no differences in relative volume of the various tissue fractions between groups or ventricles. The total volume of the ventricular freewall composed of myocytes was greater in anemic than control fetuses (LV control $5.14 \pm 0.96 \text{ cm}^3$, anemic $7.31 \pm 1.44 \text{ cm}^3$, $P<0.01$; RV control $5.02 \pm 0.94 \text{ cm}^3$, anemic $6.84 \pm 1.35 \text{ cm}^3$, $P<0.01$). Cardiomyocyte number, determined by dividing the mass of the muscular component of the ventricular freewall by average myocyte volume for that ventricle, was calculated as: control LV $0.99 \pm 0.21 \times 10^9$ cells, RV $0.57 \pm 0.14 \times 10^9$ cells; anemic LV $1.16 \pm 0.30 \times 10^9$ cells, RV $0.67 \pm 0.21 \times 10^9$ cells (NS).

Discussion

We report here for the first time the cellular changes that comprise the substantial (39%) increase in heart weight accompanying chronic fetal anemia. Consistent with the previously reported increase in cardiac output and stroke volume that occurs with chronic fetal anemia (Davis and Hohimer, 1991), we found eccentric cardiac hypertrophy that maintained wall stress. Hearts of anemic fetuses weren't edematous, and the proportions of tissue constituents (cardiomyocyte, vasculature, connective tissue) did not change. Overall, cardiomyocytes tended to be slightly larger and more mature in anemic fetuses, and we calculated that there tended to be more of them.

Other stimuli that have produced a comparable degree of cardiac enlargement include chronic pressure load (Barbera *et al.*, 2000, Jonker *et al.*, 2007a), subpressor cortisol infusion (Giraud *et al.*, 2006) and insulin-like growth factor 1 (IGF-1) infusion (Sundgren *et al.*, 2003a). In each of these cases cardiomyocyte hypertrophy, terminal differentiation and/or proliferation contributed to increased mass. We hypothesized that cardiomyocyte proliferation was responsible for accelerated cardiac growth in chronic anemia.

We ruled out cardiac edema (wet/dry ratio) or disproportionate non-myocyte growth (volume fraction) as causes of increased cardiac mass in fetal anemia, and calculated that the muscular component of the ventricular freewalls was significantly increased in mass. This difference must be the result of either larger or more cardiomyocytes, and is shown in Figure 3 as the sum volume of the mononucleated and binucleated cardiomyocytes in the ventricular freewalls. The product of number and size of binucleated cardiomyocytes was larger in both ventricles of anemic fetuses. We determined that a small degree of cellular enlargement occurred in anemic fetuses, because all cardiomyocyte length and width dimensions were greater in those animals compared to control fetuses (a result very unlikely to have occurred by chance). We could have assumed that there were approximately 39% more cardiac myocytes in anemic hearts because heart weight increased by 39%. However, we took a more careful approach to estimating cardiomyocyte number which incorporated actual measured values, and concluded that cardiomyocyte enlargement, proliferation, and terminal differentiation all contributed to accelerated growth of the anemic heart, as is natural to the near-term fetus (Jonker *et al.*, 2007b). What is unusual, then, regarding growth of the heart in the anemic fetus is not an unusual cellular response to the stimulus, but the rapidity with which normal cellular growth mechanisms were stimulated. This complex growth response may reflect multi-factorial cardiomyocyte stimulation in chronic anemia (for instance, increased coronary flow, hormonal activation and altered cardiac load), in contrast to previously described experimental models of fetal cardiac enlargement.

The balanced, proportional growth during fetal anemia is surprising when we compare it to the types of cardiomyocyte growth produced in other models of increased fetal cardiovascular stress. Right ventricular systolic pressure load and plasma protein infusion are two models of fetal hemodynamic load which result in cardiac myocytes with substantially greater dimensions (Barbera *et al.*, 2000; Jonker *et al.*, 2007a). Both of these models increased systolic arterial pressure, in contrast to slightly decreased pressures observed in anemia. The cardiac growth in this study more closely resembles that observed following infusions of sub-pressor doses of cortisol or IGF-1 (Giraud *et al.*, 2006; Sundgren *et al.*, 2003a). In contrast to anemia, IGF-1 infusion decreased the relative percent of cardiomyocytes that were binucleated as mononucleated cardiomyocytes were stimulated to proliferate. Cortisol concentrations were not different between anemic and control animals, although there was a large increase in both groups between baseline and the final day which is normal for near-term fetal sheep (Table 1). Without more intermediate samples, it is not possible to determine if cortisol increased early in the anemic sheep in this study. However, a rapid increase in plasma cortisol has been found to occur following fetal hemorrhage (Matsuda *et al.*, 1992), and premature parturition was initiated in several ewes in this study (a function of circulating cortisol levels in sheep). We propose the hypothesis that cortisol may partially mediate the cardiac growth effects of fetal anemia.

Heart tissue hypoxia is another possible modulator of cardiac growth. Hypoxia-inducible factor 1 and vascular endothelial growth factor have been shown to increase in the chronically anemic fetal sheep heart (Martin *et al.*, 1998; Mascio *et al.*, 2005). These and other factors are candidates for crosstalk between growing cardiac myocytes and coronary vasculature. However, our laboratory has shown a near-cessation of all aspects of cardiac growth following hypoxia induced by umbilicoplacental embolization (Louey *et al.*, 2007).

We do not believe that hypoxia is the primary signal driving cardiomyocyte growth in anemia, but we suspect that it is an important feature of other myocardial changes during anemic stress. While possibly not directly responsible for stimulating cardiomyocyte growth, vascular responses to tissue hypoxia profoundly alters systemic resistance and thus the loading and flow conditions of the heart.

Growth of the adult myocardium has been shown to be exquisitely sensitive to wall stress, and wall stress is likely to be very important in the fetal heart as well. The rapid hemorrhage phase of fetal anemia causes cardiac output to increase up to 50% over normal levels over the period of a few days (Davis and Hohimer, 1991). The increase in stroke volume is achieved at least in part by increasing the ventricular short axis dimension and the radius of curvature (Table 3), and thus expanding chamber volume. Increased thickness of the ventricular wall reduces wall stress according to the Law of Laplace. At our endpoint of 9 days, systolic and diastolic wall stresses were normal in anemic fetuses (Table 3), despite their hearts being much larger (Table 2). This indicates that adaptive growth of the fetal heart had already occurred by the study endpoint.

Other investigators have studied activation of intracellular signaling pathways during chronic fetal anemia at a similar gestational age (Olson *et al.*, 2006). They found that after 7 days of anemia neither total nor activated levels of Akt, JNK or p38 were increased in the fetal hearts. Phosphorylated levels of ERK were reduced in the anemic hearts compared to controls. This conjunction of anemia-induced cardiomyocyte proliferation and depressed phospho-ERK is in contrast to the necessity of ERK activation for a proliferative response to IGF-1 or angiotensin II in cultured fetal cardiomyocytes (Sundgren *et al.*, 2003a; Sundgren *et al.*, 2003b). This may be another piece of evidence that the majority of the cardiac growth and remodeling occurred earlier in the anemic period, during the phase in which hematocrit was dropping rapidly.

Clinical fetal anemia ranges from mild to profound depending on the cause, severity of affectedness, clinical treatment, and response to treatment. The degree of anemia produced in this study is comparable to that observed in human fetuses with hemolytic disease in clinical studies (Harper *et al.*, 2006; Oberhoffer *et al.*, 1999). Indeed, a guideline for serial fetal transfusion for correction of anemia dictates that fetal hemoglobin levels be allowed to fall to 4–6 standard deviations below the mean for gestational age before repeating the procedure, in order to minimize procedure-related complications (Oepkes and Van Scheltema, 2007). At an average nadir of 4.0g/100ml, the anemic fetuses in this study were 5.3 standard deviations below the control mean of 11.5 ± 1.4 g/100ml. Similar to the range of clinical anemia observed in fetuses, the gestational age of clinical onset is varied. The gestational age studied here is comparable to later onset or detection of clinical fetal anemia (Harper *et al.*, 2006; Oberhoffer *et al.*, 1999), as well as to fetuses receiving serial transfusion for correction of anemia (Oepkes and Van Scheltema, 2007). The results of this study imply that similarly anemic human fetuses have accelerated cardiomyocyte growth and maturation. These fetal adaptations may persist into adulthood as altered cardiomyocyte number, a factor important in the diseased heart.

Conclusions

We found that increased fetal cardiac mass following chronic anemia was due in part to a large and statistically significant increase in muscle volume which was accomplished by cardiomyocyte growth. We conclude that cardiomyocyte enlargement, terminal differentiation, and proliferation all contributed to this growth. Cortisol and mechanotransduction are good candidates for regulators of cardiac growth during chronic anemia, but further experiments will be necessary to establish their specific roles. The

postnatal outcomes to cardiac composition and cardiomyocyte numbers following such an episode may be altered for life and remain to be determined.

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Figure 1.

Oxygen content decreased most rapidly during the first 5 days of bleeding. Solid circles = control fetuses, open circles = anemic fetuses (n = 12 each group). Data are presented as means $\pm$ SEM.

Figure 2.

(A) Percent binucleation and (B) cell cycle activity as a percent of mononucleated myocytes (as measured by Ki67 positivity) in the hearts of anemic and control fetuses. Solid bars = control fetuses (n = 9), open bars = anemic fetuses (percent binucleation: n = 9; cell cycle activity n = 8). Data are presented as means $\pm$ SEM. *Mean significantly (p < 0.05) different from ipsilateral control.

Figure 3.

Freewall myocardial volume is composed of the total summed volumes of mononucleated and binucleated cardiomyocytes. The products of mononucleated myocyte number and volume are shown in white bars, while those of binucleated myocytes are shown in black bars. Data are presented as means $\pm$ SEM. Means significantly ($p < 0.01$) different from control fetuses for *mononucleated and binucleated cardiomyocytes combined, and †binucleated cardiomyocytes.

TABLE 1

Hemodynamic parameters in control and anemic fetuses at baseline and after 9 days of study.

	Control		Anemic		ANOVA	n (each group)
	Baseline	Final Day	Baseline	Final Day		
pH	7.38 ± 0.03	7.36 ± 0.03	7.38 ± 0.02	7.34 ± 0.05 *	P<0.01	12
P _O ₂ (mmHg)	20 ± 2	20 ± 2	20 ± 3	17 ± 1 *†	P<0.0001	12
P _{CO} ₂ (mmHg)	52 ± 3	52 ± 3	51 ± 3	51 ± 2	NS	12
Hematocrit (%)	38 ± 4	39 ± 6	35 ± 4	15 ± 2 *†	P<0.0001	13
O ₂ content (ml/100ml)	8.4 ± 1.7	8.1 ± 1.5	8.1 ± 1.7	2.3 ± 0.3 *†	P<0.0001	13
Mean Arterial Pressure (mmHg)	43.5 ± 2.9	46.9 ± 5.3	44.1 ± 2.6	42.8 ± 3.3 †	P<0.05	13
Mean Right Atrial Pressure (mmHg)	2.0 ± 0.6	2.7 ± 1.2	2.7 ± 1.1	3.6 ± 2.0	P<0.01	11
Heart Rate (bpm)	164 ± 12	147 ± 20	166 ± 17	166 ± 23	P<0.05	13
Cortisol (ng/ml)	4.7 ± 2.0	42.9 ± 21.3 *	5.0 ± 3.3	52.7 ± 22.1 *	P<0.0001	10

Means significantly (P < 0.05)

* different from baseline;

† different from age-matched controls. Data are presented as means ± SD.

TABLE 2

Fetal body and heart weights of control and anemic fetuses after 9 days of study.

	Control	Anemic
Gestational Age (days)	137 ± 1	137 ± 1
Heart Weight (g)	24.5 ± 4.1	34.0 ± 6.6 *
Body Weight (kg)	3.9 ± 0.5	4.1 ± 0.6
Heart/Body (g/kg)	6.2 ± 0.9	8.4 ± 1.4 *
LV Wet/Dry Weight	8.3 ± 2.7	7.8 ± 2.1

Each group: n = 14, except n = 3 for wet/dry weights.

* Means significantly different ($P < 0.0001$). Data are presented as means ± SD.

Ventricular dimensions, wall stress and isolated cardiac myocyte dimensions in control and anemic after 9 days of study.

TABLE 3

	Control		Anemic	
	LV	RV	LV	RV
Mid-Ventricular Radius of Curvature (mm)	5.4 ± 1.6	8.4 ± 2.7	7.5 ± 0.8 *	10.8 ± 0.6
Mid-Ventricular Wall Thickness (mm)	4.8 ± 0.7	4.4 ± 0.4	5.3 ± 0.5	5.1 ± 0.3 *
Systolic Wall Stress (mmHg)	26 ± 9	42 ± 16	31 ± 4	46 ± 5
Diastolic Wall Stress (mmHg)	2.0 ± 0.9	3.1 ± 1.0	2.7 ± 1.6	4.1 ± 2.3
Binucleated Myocyte Length (µm)	85.8 ± 5.3	91.7 ± 7.4	88.1 ± 5.8	97.4 ± 3.7 *
Binucleated Myocyte Width (µm)	13.4 ± 1.1	16.4 ± 0.9	14.3 ± 0.5	17.5 ± 1.8
Mononucleated Myocyte Length (µm)	67.0 ± 3.3	69.9 ± 5.6	68.1 ± 5.7	72.2 ± 4.8
Mononucleated Myocyte Width (µm)	11.5 ± 0.9	13.9 ± 1.0	12.2 ± 0.6	14.3 ± 1.2
Binucleated Myocyte Volume (µm ³)	6826 ± 1443	11906 ± 1995	8028 ± 862	13782 ± 2807
Mononucleated Myocyte Volume (µm ³)	3967 ± 795	6267 ± 1245	4399 ± 679	6773 ± 1265

Ventricular morphometry data n = 5 each group, isolated myocyte morphometry data n = 9 each group.

* Significantly (P < 0.05) different from ipsilateral control ventricle. Data are presented as means ± SD.

TABLE 4

Ventricular tissue fractions of hearts from control and anemic fetuses after 9 days of study.

	Control		Anemic	
	LV	RV	LV	RV
Cardiomyocyte (%)	80.7 ± 4.2	78.8 ± 4.1	81.2 ± 3.2	76.0 ± 5.6
Collagen (%)	1.3 ± 0.5	1.2 ± 0.8	1.2 ± 0.6	1.3 ± 1.2
Other Connective Tissue (%)	5.0 ± 4.4	6.1 ± 5.3	3.8 ± 2.6	5.4 ± 3.7
Vasculature (%)	13.1 ± 1.9	13.8 ± 5.5	13.8 ± 1.5	17.0 ± 3.1

Each group n = 5. Data are presented as means ± SD.