Myocyte enlargement, differentiation, and proliferation kinetics in the fetal sheep heart

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Throughout most of gestation, cardiac growth occurs by myocyte proliferation (46). Most or all cardiac myocytes lose their capacity for proliferation during the perinatal period, an event that is often marked by a final round of DNA replication without cellular division (39); in sheep, nuclear division occurs without cellular division, resulting in binucleation (12). Thus, during the latter half of gestation and in the perinatal period, there are two populations of cardiac myocytes, proliferative and nonproliferative, that contribute differently to cardiac growth. Recent studies of the cellular mechanisms of fetal cardiac growth in response to hemodynamic and hormonal challenges have uncovered the importance of these two myocyte populations in the heart of the large mammal (12, 26, 35, 48, 49). Nevertheless, basic questions about the growth and maturation of cardiac myocytes in the normal fetal heart remain unanswered.

Proliferation and terminal differentiation, the balance of which determine myocyte number, occur concurrently during the last third of gestation in sheep (17) as well as humans (2, 24, 28). Although the number of myocytes in the growing hearts of several species has been estimated (4, 9, 17, 36, 43), as has cardiac myocyte cycle activity (24, 28, 47), no attempts have been made to address the following question: How many cardiac myocytes are in the cell cycle, and what proportion of these terminally differentiate?

Enlargement of myocytes in mature hearts can be longitudinal or cross-sectional, each of which may affect the morphology of the ventricle differently (44). The fetal ventricular myocardium grows normally in proportion to expansion of the ventricular chamber (50). Cardiac myocyte volumes increase modestly during the last third of gestation in fetal sheep (17), and at least part of this enlargement is due to an expansion in cross-sectional area (46). Do fetal cardiac myocytes also enlarge in their longitudinal dimension with advancing gestational age? It has been noted that binucleated myocytes are larger than those that are mononucleated (12, 17). How much does myocyte enlargement associated with terminal differentiation contribute to overall cardiac growth?

The sheep fetus is the most frequently used model of human congenital heart and cardiovascular disease because it tolerates chronic instrumentation and experimental interventions and is of a similar size to humans at birth. An increasing number of studies have focused on how experimental interventions affect growth of cardiac myocytes in the near-term fetal sheep (12, 34, 35, 42, 48, 49, 51) and on how growth of the fetal heart influences life-long cardiovascular health in an individual (21, 23, 33, 34). This study attempts to answer some remaining fundamental questions about how the normal fetal heart grows.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee approved all animal protocols. Seventy-five time-bred ewes of mixed Western breeds were purchased from a commercial supplier. A total of 91 fetuses was studied; of these, 16 fetuses were surgically instrumented to serve as controls in unpublished, preliminary experimental proto-
cols (these instrumented fetuses spanned the gestational age range of the entire study group). Otherwise, fetuses were uninstrumented control twins of experimental fetuses or not subject to surgery whatsoever. Included were 9 singleton fetuses, 62 twins, 13 triplets, and 7 fetuses for which information was not available. Fetuses were not selected for sex, and the study group included a balance of males and females. Animals included in the study group have not been included in any previously published study.

All ewes were euthanized, as approved by the Institutional Animal Care and Use Committee, with a commercial pentobarbital sodium solution. Immediately after euthanasia of the ewe, deeply anesthetized fetuses were given 10,000 U of intravenous heparin, and their hearts were arrested in diastole with an intravenous injection of saturated potassium chloride. At autopsy, the fetuses were weighed, and their hearts were excised and trimmed in a standard manner (aortas were trimmed at the bifurcation of the common brachiocephalic artery, and pulmonary arteries were trimmed at the bifurcation of the pulmonary arteries). Their hearts were then blotted and weighed and then frozen or dissociated (see below).

Hearts from 29 fetuses were individually dissected into component parts following anatomic markers. These hearts were not dissociated because dissociation changes free wall weight and an intact myocardium is necessary for perfusion. The atria were dissected from the ventricles at the level of the annulus of the respective atrioventricular valve and freed from the cavae and pulmonary veins. The atria were divided such that one-half of the intra-atrial septum was allocated to each atrium. The ventricular free walls were dissected free from the septum such that the septum retained a border of epicardial myocardium equal to its thickness. The great vessels were then freed from the ventricles just below the semilunar valves. After they were weighed, component parts were frozen in liquid nitrogen and stored at −80°C.

Hearts from 63 fetuses were enzymatically dissociated as previously described, with some modifications (12). Briefly, the aorta was cannulated without permitting air to enter the coronary arteries. Tyrode solution (in mM: 140 NaCl, 5 KCl, 1 MgCl2, 10 dextrose, and 10 HEPES, pH adjusted to 7.35 with NaOH) was perfused through the coronary circulation until the myocardium had become blanched, and then Tyrode solution with 160 U/ml Worthington type II collagenase (Worthington Biochemicals, Lakewood, NJ) and 0.78 U/ml protease type XIV (Sigma, St. Louis, MO) was perfused for ~5 min. Calcium-free Kraftbrühe buffer (in mM: 74 l-glutamic acid, 30 KCl, 30 KH2PO4, 20 taurine, 3 MgSO4, 0.5 EGTA, 10 HEPES, and 10 dextrose, pH adjusted to 7.37 with KOH) was perfused through the heart to rinse out the collagenase. All perfusion solutions were preheated to 39°C and bubbled with a 95% O2-5% CO2 gas mixture. The right (RV) and left ventricular (LV) free walls were then individually dissected free (as above), scored with a blade, and gently agitated in Kraftbrühe buffer to loosen myocytes. The myocyte slurry was set aside at room temperature for 30 min and thenfixed with an equal volume of fresh 2% formaldehyde in PBS.

Myocyte Measurements

**Myocyte morphometry.** The long-axis lengths and maximal perpendicular widths of dissociated myocytes were measured from digital photomicrographs as previously described (12). Briefly, fixed myocytes were prepared in a wet mount with methylene blue, selected by a random, nonrepeating method, and photographed with the use of a ×40 objective on a light microscope (Zeiss Axiohot, Bartels and Stout, Bellevue, WA). Photomicrographs were analyzed by calibrated software (Optimas, Seattle, WA). At least 100 cells were measured per ventricle. In the youngest fetuses, binculated myocytes were rare, but no fewer than five binculated myocytes were included for each sample.

Myocyte volumes have been calculated previously by correcting from the volume of a cylinder with a height equal to the length of the myocyte and a diameter equal to the myocyte width (12). In the present study, a correction factor was calculated to more accurately estimate myocyte volume. 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. Measurements were taken from binculated and mononucleated myocytes (n = 9–12 each) from both ventricles of 35 fetuses evenly distributed between 90 and 145 days of gestational age (dGA). Myocytes were found to be more spindle shaped in the younger fetuses; thus the correction factors were found to change with gestational age (Table 1).

**Number of nuclei per myocyte.** Random, nonrepeating fields from a wet mount of fixed cardiac myocytes stained with methylene blue were visualized on a light microscope. The numbers of myocytes in each field with one, two, or more nuclei were counted. No fewer than 300 myocytes from each ventricle of each animal were counted to determine the proportion of nucleation.

**Cell cycle activity.** Cell cycle activity was detected by the Ki-67 antibody MIB-1 (DAKO, Carpinteria, CA). Dissociated myocytes were dried on Superfrost Plus slides, postfixed in cold acetone for 30 min, and then boiled in 10 mM sodium citrate (pH 6.0) for 6 min. Endogenous peroxidase activity was blocked by incubating the slides for 5 min with 0.03% hydrogen peroxide, and nonspecific binding was blocked by incubation in 1% BSA with 0.5% Triton X-100. Slides were then incubated at 4°C overnight with a 1:200 dilution of the Ki-67 antibody. The secondary antibody (biotinylated anti-mouse IgG; Vector Laboratories, Burlingame, CA) was applied to the slides at a dilution of 1:200 and left at room temperature for 60 min. Incubation with avidin-biotin complex (Vectastain ABC kit; Vector Laboratories) followed for 60 min at room temperature. Positive cells developed dark brown nuclei on incubation with diaminobenzidine. All incubation steps were followed by three washes in PBS. Cells were lightly counterstained with methylene blue and dehydrated, and the slides were mounted with coverslips. Fields of stained myocytes were selected for counting as for the determination of percent nucleation. At least 500 cells were counted per ventricle per fetus for cell cycle activity analysis.

Myocytes that stained positive with diaminobenzidine were counted according to their morphology. Six categories of positive myocytes were considered: 1) mononucleated myocytes similar in morphology to nonpositive mononucleated myocytes (Fig. 1C), 2) binculated myocytes without a cleavage furrow (similar in morphology to nonpositive binculated myocytes; Fig. 1D), 3) myocytes with four nuclei without a cleavage furrow (similar in morphology to nonpositive myocytes with 4 nuclei; Fig. 1G), 4) pairs of sister cells undergoing cytokinesis, evidenced by the presence of a cleavage furrow (Fig. 1E), 5) myocytes similar in morphology to cells undergoing cytokinesis but lacking a conjoined sister cell (Fig. 1F), and 6) myocytes with two cleavage furrows separating a central binculated myocyte from two peripheral mononucleated myocytes (Fig. 1H). The morphologies of myocytes in categories 4–6 were always associated with positive Ki-67 staining.

Calculations

**Terminology.** Whereas cell numbers provide information on cell division rates, staining of cells with the Ki-67 antibody provides information only on nuclear division rates. Nuclear division (karyokinesis) can be followed by cellular division (cytokinesis), producing two mononucleated cells from a single mononucleated precursor, or, if not followed by cellular division, merely transforms a formerly mononucleated cell into a binculate without an increase in total cell number. All calculations were made separately for the LV and RV free walls. The mean weight of the ventricular free wall at each age between 94 and 146dGA was found by multiplying the mass ratio of the ventricles to the heart (Table 1) by the heart weight (calculated from Table 1). To correct for the estimated fraction of the ventricular free wall that is composed of nonmyocyte tissue, this weight was
multiplied by a correction factor of 0.78 for the LV and 0.795 for the RV (46). This yielded the weight of the ventricular free wall composed of myocytes (W

The number of cells (N\text{total}) was calculated by dividing the total weight of the ventricular free wall in grams by the volumes of the cells in milliliters (see Fig. 5). At 95dGA, essentially all cells were mononucleated (Fig. 6), but later in gestation we took into account the fractions of mononucleated (F

Because binucleated myocytes are larger than mononucleated myocytes, the contribution of terminal differentiation was calculated as the difference in myocyte volume between binucleated and mononucleated cells (thus accounting for the “loss” of a mononucleated myocyte):

\[\text{Dbi}_{GA} \times (\text{V}_{bi,GA} - \text{V}_{mon,GA})\]
Fig. 1. Cardiac myocytes from the fetal sheep with immunofluorescence staining for myosin (red; primary antibody Abcam ab15) and a Hoechst nuclear marker (cyan). Myocytes were also probed with an antibody against Ki-67 (diaminobenzidine staining shown in yellow for contrast). A: mononucleated myocytes (arrows). B: binucleated myocytes (arrows) and a myocyte with 4 nuclei (double-headed arrow). C: mononucleated myocyte positive for Ki-67 (arrow). D: myocyte containing 2 nuclei positive for Ki-67 (arrow). E: dividing myocyte positive for Ki-67; a cleavage furrow is clearly visible (arrowhead). F: myocyte positive for Ki-67 with the same morphology as a dividing myocyte (shown in E) but detached from the sister myocyte (arrow). G: myocyte with 4 nuclei positive for Ki-67 (arrow). H: myocyte with 2 cleavage furrows (arrowheads) producing 2 mononucleated daughter cells (ends) and 1 binucleated myocyte (arrow). Scale indicated in H is accurate for all panels.
Daily mass gain due to enlargement of mononucleated myocytes was equal to the number of mononucleated myocytes multiplied by one-half of the difference in myocyte volume between the preceding and succeeding days:

\[ V_{\text{mon GA}} \times 0.5 \times (V_{\text{mon GA+1}} - V_{\text{mon GA}}) \] (8)

The daily growth increment due to enlargement of binucleated myocytes was likewise estimated.

Finally, the ability to identify cells that are in the process of nuclear division by staining for the Ki-67 antigen enables us to calculate the number of myocytes in the cell cycle. The proportions of stained cells were obtained from the regressions in Fig. 7 (also in Table 1). The number of myocytes in the cell cycle \((N_{\text{ki}})\) was found by multiplying the proportion of myocytes that stained for Ki-67 \((\%_{\text{ki}})\) by the total number of myocytes \((N_{\text{total}})\):

\[ N_{\text{ki GA}} = \%_{\text{ki GA}} \times N_{\text{total}} \] (9)

Cardiac Myocyte Apoptosis

Frozen LV and RV myocardium from nine fetuses was embedded in OCT (Tissue-Tek, Torrance, CA) and cryosectioned at a thickness of 5 μm. Fluorescent DNA-binding dye Hoechst 33258 was used to determine nuclear chromatin morphology as a quantitative index of apoptosis as described previously (10). The tissue slices were stained with Hoechst 33258 (5 μg/ml) for 10 min, and the nuclear morphology was examined by fluorescence microscopy with the SPOT digital camera imaging system. Cells were scored as apoptotic if they showed unequivocal nuclear chromatin condensation and fragmentation, as in positive controls of fetal rat hearts at 21dGA. Sample identity was concealed during scoring. To quantify apoptosis, an average of 2,080 nuclei from each sample were examined.

Statistical Analysis

Linear regression was used to determine the relationships between fetal weights, myocyte dimensions, and cell cycle activity and gestational age. Nonlinear regression was used to determine the relationship between the changing proportion of mononucleated myocytes with gestational age (Boltzmann sigmoidal equation). Unpaired t-tests were used to compare parameters from groups of singleton and twin fetuses. Fisher’s exact test was employed to determine whether the sex ratio was different between groups in the comparison of singleton and twin fetuses. Statistical analysis was performed with Graphpad Prism version 4.0a for Mac OSX (Graphpad Software, San Diego, CA). \(P < 0.05\) was considered significant.

RESULTS

During the last third of gestation, fetal sheep underwent exponential growth of heart and body (Fig. 2). LV-to-heart mass ratio did not change with advancing gestational age, but that of the RV to heart increased slightly during the same period (Table 1 and Fig. 3).

Lengths and widths of myocytes from the fetal ventricles over the last third of gestation are shown in Fig. 4. The lengths of binucleated myocytes from both ventricles and the lengths of RV mononucleated myocytes did not increase significantly with gestational age (Table 1). LV mononucleated myocytes significantly increased in length but only slightly. Binucleated and mononucleated myocytes from both ventricular free walls increased in width during this period. Accordingly, the ratio of lengths to widths decreased with advancing age in mononucleated and binucleated myocytes of both ventricles (Table 1).

Myocyte volumes, calculated from myocyte dimensions and a correction factor, increased with advancing gestational age (Fig. 5). Binucleated myocytes were longer and wider than mononucleated myocytes, and RV myocytes were longer and wider than LV myocytes (Fig. 4).

Ventricular myocytes were nearly 100% mononucleated until 100dGA, after which the proportion that were mononucleated decreased (Fig. 6, A and B), whereas the proportion that were binucleated increased. At 135dGA, the myocyte populations of both ventricles were ~50% mononucleated. Ages 135–145dGA corresponded to the period with the greatest degree of variability in percent mononucleation between fetuses. However, within this age range, the percent mononucleation within fetuses correlated well between LV and RV (Pearson’s \(r = 0.8538\), \(P < 0.0001\), \(n = 19\)). A very small proportion of myocytes in the fetal sheep heart were found to have four or, very rarely, three nuclei (Fig. 6, C and D). These occurred most commonly after 135dGA and typically accounted for 1% or less of all myocytes. Myocytes with four nuclei are common in the ventricles of adult sheep (Jonker, unpublished observations).

Myocytes determined to be in the cell cycle, as assessed by Ki-67 immunostaining, were predominantly mononucleated (as shown in Fig. 1C). The second most common type of Ki-67-positive myocyte was fixed in the processes of cytokinesis, as determined by the presence of a cleavage furrow (Fig. 1E). Some Ki-67-positive myocytes appeared to belong in this second category, except that they lacked a conjoined sister cell (Fig. 1F). These myocytes may have just concluded cytokinesis in vivo, or the sister cells may have been separated during or after myocyte isolation and fixation. A small proportion of myocytes positive for Ki-67 contained two nuclei but no
cleavage furrow (Fig. 1D). For the purpose of considerations of cell cycle activity, all of these were considered the final stages of a single dividing mononucleated cell and were accordingly counted as a single myocyte. Very rarely a Ki-67-positive myocyte would be found with four nuclei (Fig. 1, G and H). If cleavage furrows were present, they always separated a central binucleated myocyte from two mononucleated cells. This morphology of myocyte cytokinesis has been described before in chick cardiac myocytes (32). In multinucleated myocytes, either all or none of the nuclei stained positive for the Ki-67 antigen.

Cell cycle activity was found to be quite variable in cardiac myocytes during the last third of gestation (Fig. 7). Nevertheless, cell cycle activity decreased in both ventricles with advancing gestational age (Table 1). At 95dGA, an average of ~7% of cardiac myocytes was in the cell cycle, as assessed by Ki-67 immunostaining. By 140dGA, only 1.5% LV myocytes and 3% LV myocytes were in the cell cycle (Fig. 7). Despite variability between fetuses, cell cycle activity was correlated between the LV and RV of each fetus (135–145dGA; Pearson’s r = 0.5395, P < 0.02, n = 19).

The numbers of cardiac myocytes in the ventricular free walls were calculated for the last third of gestation (Fig. 8, A and B). At 95dGA, there were $6.8 \times 10^8$ mononucleated and very few binucleated myocytes in the LV free wall. The number of mononucleated myocytes peaked at $8.1 \times 10^8$ cells near 115dGA, at which time there were $9.0 \times 10^8$ binucleated myocytes (9 x $10^8$ myocytes in total). The number of mononucleated myocytes declined to $5.6 \times 10^8$ by 140dGA, whereas binucleated myocytes increased in number to $7.3 \times 10^8$ (1.3 x $10^9$ myocytes in total).

At 95dGA, there were $2.8 \times 10^8$ mononucleated myocytes and very few binucleated myocytes in the RV free wall. The number of mononucleated myocytes peaked at $4.4 \times 10^8$ at 120dGA, at which time there were $1.1 \times 10^9$ binucleated myocytes ($5.5 \times 10^8$ myocytes in total). At 140dGA, the RV contained $3.0 \times 10^8$ mononucleated myocytes and $4.5 \times 10^8$ binucleated myocytes ($7.5 \times 10^8$ myocytes in total).

The numbers of myocytes active in the cell cycle in the ventricular free walls were calculated (Fig. 8C). At 95dGA, there were $4.9 \times 10^7$ such cells in the LV. This number declined steadily to $2.0 \times 10^7$ myocytes at 140dGA. In the RV, the number of myocytes active in the cell cycle was remarkably constant at $1.9-2.4 \times 10^7$ myocytes between 95 and 140dGA.

The fraction of mononucleated myocytes that were active in the cell cycle that terminally differentiated (rather than proliferated) was calculated (Fig. 8D). At 95dGA, very few myocytes were becoming binucleated in either ventricle. By 120dGA, 60% of myocyte cell cycle activity in the LV ended in terminal differentiation, whereas in the RV this value was only 45%. At 140dGA, 54% cell cycle activity in the LV ended in terminal differentiation, and in the RV that value was 70%.

The proportions of apoptotic cells were measured in tissue sections from LV and RV of nine fetuses (3 at each of ~95dGA, 111dGA, and 139dGA). No myocardial nucleus from an average of 2,080 nuclei inspected from each sample was found to display the typical nuclear morphological characteristics of apoptosis. This suggests that <0.05% nuclei are apoptotic in fetal sheep hearts at the three gestational ages examined.

Singleton and twin fetuses between 135 and 140dGA were compared to determine differences in heart growth. These groups were sex balanced and were of the same mean age (Table 2). Singleton fetuses were 22% heavier and had 38% larger hearts than twin fetuses. The heart-to-body mass ratio was not significantly different between singleton and twin fetuses. Myocytes from singleton fetuses were compared with myocytes from the ipsilateral ventricle of twin fetuses. No differences were found between singletons and twins in either mononucleated or binucleated myocyte lengths or widths in either ventricle. The proportions of myocytes that were mononucleated were not different between singleton and twin fetuses in either ventricle. The proportions of myocytes that were active in the cell cycle were also not different between singleton and twin fetuses in either ventricle, although there was a trend for twin fetuses to have less LV cell cycle activity than singleton fetuses (LV $P = 0.0565$, RV $P = 0.3028$).

**DISCUSSION**

*Myocyte Cycle Activity: Proliferation and Terminal Differentiation*

Cellular proliferation is recognized as a primary means of cardiac growth during embryonic and fetal life. Indeed, the number of myocytes in the heart more than doubled between 95 and 145dGA (Fig. 8). However, proliferative growth was not responsible for the exponential cardiac growth during the last third of gestation. Rather, the contribution of proliferation was relatively constant (especially in the RV), and consequently a proportion of daily free wall growth became smaller with advancing gestational age (Fig. 9).

Proliferation is but one outcome of myocyte cell cycle activity. As the fetuses grew older and matured, the outcome shifted from karyokinesis followed by cytokinesis (proliferation) to karyokinesis alone (terminal differentiation) (Fig. 8D). As more than 50% of the myocytes undergoing nuclear division terminally differentiated (Fig. 8D; LV: 115 days), the total number of mononucleated cells started to decline (Fig. 8A; LV: 115 days). The number of binucleated cells increased rapidly from ~115 to 120dGA at the expense of the mononucleated myocyte population (Fig. 8, A and B). These changes in myocyte numbers, driven by both proliferation and terminal...
differentiation, are reflected in the declining percentage of myocytes that are mononucleated (Fig. 6).

Few similarly quantitative studies of cardiac myocyte cycle activity across late gestation have been carried out in humans or in any other large mammal. A similar sequence of proliferation, terminal differentiation, and myocyte enlargement occurs in the canine as in the sheep, although terminal differentiation occurs postnatally in the canine, as in other altricial species (14). Investigations of postmortem human fetal hearts using PCNA, Ki-67, and morphological scoring of nuclei have resulted in measurements of cell cycle activity that are variable and decline with advancing gestational age (22, 24, 28). The results of our study suggest that the decrease in myocyte cycle activity with increasing maturity is due to a declining proportion of myocytes capable of proliferation.

Fig. 4. Lengths (A and B) and widths (C and D) of fetal sheep cardiac myocytes throughout the last third of gestation (n = 63 fetuses in each group; at least 100 myocytes were measured per ventricle of each fetus). The best-fit linear regression lines and 95% confidence intervals of each data set are shown; regression values are included in Table 1.

Fig. 5. Calculated volumes of fetal sheep cardiac myocytes throughout the last third of gestation (n = 63 fetuses in each group) in left (A) and right (B) ventricle. The best-fit linear regression lines and 95% confidence intervals of each data set are shown; regression values are included in Table 1.
We found a high level of interanimal variability in myocyte cycle activity (Fig. 7), a characteristic of cardiac myocytes that has been noted before and explained as a characteristic of highly differentiated tissues (43). If proliferation occurs in intermittent bursts, then interanimal samples of cell cycle activity will be highly variable, as was observed. Time-lapse photographic studies of fetal rat cardiac myocytes in culture have shown that sister cells tend to have similar intervals between mitotic events (13, 18). An alternative to an intrinsic

Fig. 6. Proportions of cardiac myocytes with 1, 2, or more nuclei during the last third of gestation (n = 63 fetuses, at least 500 myocytes per ventricle of each fetus were counted). A and B: the proportion of mononucleated myocytes begins to decrease after ~100 days of gestational age. Most of these myocytes become binucleated. C and D: a very small proportion of myocytes have 4 nuclei (or, extremely rarely, 3 nuclei) during fetal life. Shown are Boltzmann’s sigmoidal equations fit to each data set and the 95% confidence intervals of each data set are shown; regression values are included in Table 1.

Fig. 7. The proportion of all cardiac myocytes in the cell cycle decreases toward term (n = 63 fetuses, at least 500 myocytes per ventricle of each fetus were counted). A: left ventricle. B: right ventricle. Insets: cell cycle activity normalized to the proportion of myocytes that are mononucleated and thus capable of proliferating or becoming terminally differentiated. The best-fit linear regression lines and 95% confidence intervals of each data set are shown; regression values are included in Table 1.
synchrony, stimulation of cardiac growth in vivo, could well occur intermittently. Fetal and maternal sheep display diurnal rhythms that may regulate fetal cardiac growth (20, 31, 37, 45). This hypothesis is supported by our observation that cell cycle activity was correlated between the LV and RV within each fetus.

Kinetics of the Fetal Sheep Cardiac Myocyte Cell Cycle

We were interested to compute how often an average mononucleated myocyte goes into nuclear division (to undergo proliferation or binucleation). We considered that, if, for example, the number of cells that completed karyokinesis during the given day was 5% of all mononucleated cells, the average interval between nuclear divisions had to be 20 days. The resting period of the mononucleated cell would then be 20 days minus the duration of the division process, but, for simplicity, we report the average interval (e.g., 20 days) in Fig. 10. Similarly, we calculated the duration of a cell cycle based on the consideration that if, for instance, 5% of all mononucleated cells completed a nuclear division on the day of interest, but 10% of all mononucleated cells on that day stained with the Ki-67 antibody, the cells in nuclear division must remain stained for an average of 2 days.

The results of our calculations of the interval between nuclear divisions and the duration of cell cycle activity in fetal cardiac cell were presented in Fig. 8. Calculated total number of cardiac myocytes increased throughout the last third of gestation in the left (A) and right (B) ventricle. The number of binucleated myocytes increased at the expense of mononucleated myocytes, which declined in number after ~115 days of gestational age in the left and ~125 days of gestational age in the right ventricle. C: the number of myocytes in the cell cycle declined rapidly in the left ventricle after ~105 days of gestational age and declined in the right ventricle after ~125 days of gestational age. D: the proportion of myocytes becoming binucleated (rather than proliferating) increased rapidly after ~100 days of gestational age.

Table 2. Differences between near-term twin and singleton fetuses

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<th>Singleton (n = 5)</th>
<th>Twin (n = 8)</th>
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<tr>
<td>Sex (M/F)</td>
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<tr>
<td>Gestational age (range 135–140 days)</td>
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<td>Body weight, kg</td>
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<td>Heart weight, g</td>
<td>37.4±7.1</td>
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<td>Mononucleated myocytes (% of all myocytes)</td>
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<td>Myocyte cell cycle activity (% of all myocytes)</td>
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<td>3.5±1.6</td>
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Values are mean ± SD. M/F, male/female. *Different from singleton, P < 0.05.
myocytes are surprising. Over the last third of gestation, the interval between nuclear divisions became shorter (Fig. 10). Concurrently, cell cycle duration decreased (thus the number of myocytes in the cell cycle stayed the same or decreased, as shown in Fig. 8C). The decline that we found in cell cycle duration in sheep seems to contradict findings in rats, mice, and chicks that indicate there is a “considerable protraction of the mitotic cycle phases with a progressive differentiation of cardiomyocytes” (43). Consideration of several factors may lend insight. First, we cannot separate the kinetics of terminal differentiation from those of proliferation (the duration of activity and the interval between periods of cell cycle activity may not be the same for terminal differentiation and proliferation). Second, fundamental developmental differences in cardiac myocyte cell cycle kinetics may exist between small mammals with high metabolic rates and short gestations and lifespans and large mammals with slower metabolic rates and longer gestations and lifespans. Third, differences between our calculations and those of other investigators may reflect the assumptions made in the respective models.

One assumption, implicit in these calculations, is that all mononucleated myocytes, and only mononucleated myocytes, are equally competent to enter the cell cycle. Myocytes in the cell cycle expressed as a proportion of mononucleated cells were nearly constant during the last third of gestation (Fig. 7, inset), supporting the idea that the mononucleated cells were the source of myocytes entering the cell cycle. Including the entire mononucleated myocyte population as equally competent to enter the cell cycle is necessary because we have no evidence that hyperplasia is limited to a more discrete population. Indeed, studies have suggested that, although proliferative myocytes do not all have the same generative potential, the generation of new cells is not the sole provenance of a subset of specialized cardiac myocytes (13, 18).

Myocyte Apoptosis

Programmed cell death, or apoptosis, is a feature of the development of the embryonic heart (1, 41) and also occurs in the myocytes of near-term rat hearts at a frequency of 1.4–2% (10, 53). Our finding suggests that apoptosis occurs in <0.05% of cardiac nuclei in fetal sheep in the last third of gestation. Similar findings were obtained by transferase-mediated dUTP nick end labeling staining of cardiac tissue sections of fetal sheep (Jonker, unpublished observations). These results are consistent with other studies in newborn sheep (19, 27, 29) and human fetuses (22). Apoptosis may play a minor role in the normal development of the large mammal during the final third of gestation, and it is unlikely that a very low rate of apoptosis would affect our calculations substantially.
Myocyte Enlargement

Cardiac myocyte width (Fig. 4, C and D) (46) and volume (Fig. 5) (17) increased during gestation in the sheep fetus. We found that cardiac myocytes for the most part did not increase in length during the last third of gestation (Fig. 4, A and B). The contribution of enlargement of mononucleated cardiac myocytes to daily free wall growth was relatively constant, and consequently a proportion of total growth became smaller with advancing gestation. In contrast to the large contribution of myocyte enlargement to growth of the postnatal heart, enlargement of binucleated myocytes contributed only a small amount to overall growth, and only near term.

Terminal differentiation is associated with myocyte enlargement, as binucleated myocytes are substantially larger than mononucleated myocytes. Enlargement associated with terminal differentiation involved both myocyte lengthening and an increase in cross-sectional size of greater magnitude than the gradual growth of either mononucleated or binucleated myocytes during the last third of gestation. Consequently, during the last third of gestation, the myocyte enlargement due to terminal differentiation was responsible for a large proportion of total ventricular growth (Fig. 9).

Singleton and Twin Fetuses

Differences between singleton and twin fetal sheep in regards to body and heart weight have been noted previously (25). Comparison of singleton and twin fetuses (selected randomly for being the larger or smaller of the twin pair) reveals that, although the bodies and hearts of twins are smaller than those of singletons, there are no differences in myocyte sizes or the proportion of myocytes that are mononucleated (Table 2). Although not statistically significant, there is a trend for cell cycle activity to be lower in twins than singletons. Because the hearts of singletons are heavier than those of twins, but myocyte size is the same between the two, it is likely that singletons have more myocytes than twins. Similar findings have been noted in rats, where differences in cardiac myocyte number at weaning are known to persist into adulthood (11, 15).

Differences Between the LV and RV

It is apparent from the data shown in this and other studies (17, 46) that there are differences in the normally growing sheep fetus between the myocytes of the LV and RV. It is uncertain why the fetal RV has fewer, larger myocytes (Figs. 4 and 5). The teleological explanation that the fetal RV is preparing for its postnatal role as the pump for the low-resistance pulmonary circuit does not provide insight into the regulatory mechanisms that determine growth of the fetal cardiac myocytes. It is possible that, despite the anatomic specializations that enable the fetal ventricles to pump in parallel, differences in wall stress between the two ventricles influence mechanical stress-controlled myocyte growth regulation (5, 6, 40). Another possibility is that the LV and RV myocytes are intrinsically different from each other, as the LV and the RV myocardium are derived from different sources of progenitor cells (16), and myocyte properties can differ between the two ventricles (30, 52).

Myocyte Number in the Postnatal Heart

We found that myocyte number was still increasing slowly in the near-term fetal heart and presumably continues to increase slowly for some period in the neonatal heart. Birth is a landmark event, after which the growth milieu and demands on the heart differ from those during fetal life. Therefore, the relationships found between myocyte cycle activity, terminal differentiation, and so forth, and gestational age cannot be extrapolated into neonatal life. Although the proportion of myocytes in the cell cycle that become terminally differentiated remains above 50%, the number of mononucleated myocytes in the heart will continue to decline.

In the adult human, myocyte number is correlated with the capacity of the heart to adapt to chronically increased hemodynamic load (3). A limitation of this observation is that morphological estimates of myocyte number in the adult human heart cannot determine the period during which myocyte number was set, whether in adulthood or in immaturity. The de novo generation of adult cardiac myocytes is the topic of debate (8, 38), but the proposed mechanism differs from that which occurs in the fetal heart. In this model, adult cardiac stem cells continuously replenish a pool of transiently prolif-
erating myocytes that terminally differentiate into the adult cardiac myocyte phenotype (7). The role of cardiac stem cells in the fetal heart was not investigated in this study.

Limitations of This Study

Burrell et al. (17) recently measured myocyte sizes and calculated the number of myocytes in the ventricular free walls of fetal sheep. Their estimates of cardiac myocyte size are similar to ours. Although their estimates of cardiac myocyte number are approximately twice the numbers calculated here, our trends with advancing gestational age are very similar. We feel that the differences in myocyte number estimated can be accounted for by methodological differences. We corrected our ventricular masses by the proportion of the myocardium composed of nonmyocyte tissue (46), whereas Burrell et al. chose not to make this correction. Furthermore, our fetuses were drawn primarily from twin pregnancies, whereas Burrell et al. primarily used singleton fetuses. These methodological differences would tend to increase the cell number estimate in Burrell et al. compared with ours; thus we feel that there is no conflict between the data of Burrell et al. and our data.

Conclusions

During the last third of gestation, the myocyte mass of the fetal sheep heart increased by proliferation, enlargement of mononucleated and binucleated myocytes, and enlargement of myocytes as they underwent terminal differentiation (Fig. 9). It is remarkable that these processes are so well coordinated that growth of the heart remained closely matched to growth of the body during this period (Fig. 2). The contributions to growth of proliferation and enlargement of mononucleated myocytes were relatively constant and proportionately much larger earlier than later in gestation. Enlargement of binucleated cardiac myocytes contributed only modestly and only near term. Neither mononucleated nor binucleated myocytes increased in length during the last third of gestation, although increases in myocyte cross-sectional diameter caused myocyte volumes to increase throughout this period. Terminal differentiation became the more frequent outcome of myocyte cell cycle activity after ~115dGA, after which the numbers of mononucleated myocytes declined. The increase in myocyte size associated with the transition from mononucleation to binucleation (terminal differentiation) became a surprisingly large, and hitherto unrecognized, proportion of daily free wall mass gain with advancing age. Both the interval between nuclear divisions and the duration of cell cycle activity declined substantially during this same period. The duration of the cardiac myocyte cycle in the fetal large mammal warrants further investigation into earlier as well as later stages. These data on normal cardiac growth may enable a more detailed understanding of the consequences of experimental and pathological interventions in prenatal life.

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