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Biochemical Correlates of Cardiac Hypertrophy

IV. OBSERVATIONS ON THE CELLULAR ORGANIZATION OF GROWTH DURING MYOCARDIAL HYPERTROPHY IN THE RAT

By D. Grove, B.S., R. Zak, Ph.D., K. G. Nair, M.D., Ph.D.,
and V. Aschenbrenner, Ph.D.

ABSTRACT

The mechanisms by which the DNA content of the heart increases following acutely induced cardiac hypertrophy were investigated in mature Sprague-Dawley rats. Special attention was given to the cellular organization of the growth process. Autoradiographic studies provided conclusive evidence that the uptake of tritiated thymidine is completely limited to nonmuscular cellular elements, chiefly connective tissue cells. The frequency of labeled nuclei was increased by sixfold during hypertrophy. The thymidine pool was not appreciably different in the hypertrophied hearts. Connective tissue nuclei formed a larger proportion of the total nuclear population in hypertrophied hearts, and their distribution was less uniform than in the normal heart. Quantitative histologic studies also showed that the total number of left ventricular muscle cell nuclei did not increase during hypertrophy but rather may have decreased slightly. Both the concentration and the total amount of hydroxyproline increased in parallel with the proliferative changes in the connective tissue and provide further supportive evidence to the autoradiographic and histologic studies.

ADDITIONAL KEY WORDS aortic banding autoradiography
DNA hydroxyproline content quantitative histology
thymidine pool

■ In the preceding paper (1) we reported that in hearts hypertrophying in response to experimentally produced aortic constriction the DNA content increases proportionally to the increase in heart weight in the early phase of hypertrophy. (The term cardiac hyper-

trophy will be used to mean cardiac enlargement without regard to possible hyperplasia.) In the chronic phase of hypertrophy, the DNA content falls below the level present in the acute phase but is still higher than in control animals. Although we found that polyploid frequencies in muscle nuclei more than doubled, the large increase in DNA content in hypertrophied hearts could not be accounted for by the increased polyploidy. No mitotic activity was observed in differentiated muscle cells, but the mitotic index in nonmuscular elements was greatly increased.

These results indicate strongly that cell proliferation is predominantly, if not exclusively, limited to nonmuscular cells at least in the model of rapidly developing cardiac hypertrophy which was under examination. Similar results and conclusions have been obtained by Crane and Dutta (2), Meerson

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and Alekhina (3) and Morkin and Ashford (4).

The increased cardiac muscle mass after aortic constriction therefore results in the development of larger muscle cells, in which the ratio of the volume of the cytoplasm to the volume of nucleus is increased. Meerson has suggested that the decrease in DNA relative to cell volume may cause a deficiency in transcriptive capacity and therefore lead to decreased cardiac function (5). There is as yet, however, no evidence that deficiency of sites for transcription in nuclear DNA can limit the process of normal protein synthesis and cell function. Yaffe and Feldman's observation that messenger RNA for muscle proteins has a relatively long life is evidence against this view (6). Furthermore, the possible existence of the mechanism of gene amplification in differentiated cells as suggested by Pelc (7) and demonstrated for cistrons for ribosomal RNA in frogs (8) could overcome possible deficiencies in transcription sites.

In the present paper we examine further the processes of DNA synthesis and cell proliferation in the adult rat heart during rapid growth induced by an increased work load. A change in tissue architecture is discussed on the basis of results obtained by autoradiography, quantitative histological methods, detailed statistical analysis of nuclear populations in heart muscle, and chemical determination of hydroxyproline content.

Materials and Methods

Supravalvular aortic stenosis was produced in female Sprague-Dawley rats weighing 200 to 220 g as described by Nair et al. (9). Normal and sham-operated litter mates served as controls. The details of the preparation of the tissue specimens for biochemical and histological analysis are given in the previous paper (1).

AUTORADIOGRAPHY

The autoradiographic techniques were based on those developed by Messier and Leblond (10) and Joftes (11).

Two hundred microcuries of tritiated thymidine (specific activity 6 $\mu\text{c}/\text{mole}$) were injected intraperitoneally 48 hours after operation. Animals were decapitated 24 hours later under ether anesthesia, and their hearts were removed, fixed,

and mounted in paraffin blocks. Four-micron sections were cut and mounted on glass slides. They were then dipped in Kodak NTB-2 liquid emulsion at 42°C, dried in absolute darkness for 1 hour, and exposed for 1 to 3 weeks at 4°C in light-proof boxes containing Drierite. Sections were developed for 2 minutes and 45 seconds in Kodak D-19 developer at 19 \pm 0.5°C, stopped by dipping in water for 10 seconds, and fixed in Kodak Acid Fixer (prepared in distilled water) for 2 minutes. Slides were washed and stained with hematoxylin and eosin B, or with cold azure B, pH 4. They were dehydrated and mounted under oil of refractive index 1.540. The frequencies of labeled nuclei were determined by counting, in each heart, 100 labeled nuclei or 500 fields, whichever came first.

AUTORADIOGRAPHIC GRAIN COUNTING

Grains in autoradiographs were evaluated in a probabilistic manner. An eyepiece insert was prepared for the microscope, which made it possible to determine various standard areas in each field. The possible standard areas (all either square or rectangular in shape) were 5, 10, 15, 25, 50, 75, 100, 200, or 400 units, and for the whole field, 12,000 units (equal to about 0.015 mm² with the ocular used). For each autoradiograph to be counted, 20 random fields were selected, and all the grains in each field, whether apparently associated with labeled nuclei or not, were counted. For the total number of grains thus counted, the average number of grains associated with each of the standard field areas could be calculated. Each of these numbers was considered to approximate the Poisson parameter λ , from which the background (random) likelihood of finding any number of grains in each standard area could be determined. The grains were then counted, and the number of grains, the smallest of the standard areas able to circumscribe all the grains associated with the nucleus, and the cell type were determined for each apparently labeled nucleus that was observed. After a number of nuclei in randomly selected fields had been counted, a table listing the number of events required to exceed Poisson predictions of probabilities for various values of λ was consulted, and all nuclei having as many grains or more than the number listed for a probability of 10⁻⁴ at the appropriate λ value were considered labeled.

STAINING

Hearts to be studied by microscopic methods were prepared in a standard manner. Animals were killed by a blow on the head, and their hearts were excised, trimmed to remove the atria and external connective tissue, and weighed. After weighing, the ventricles were fixed in phosphate-buffer 12% formaldehyde, pH 6.9, for

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24 hours. They were then cut in half by a slice at a right angle to the long axis of the left ventricle, approximately at the midpoint of the axis, and fixed 24 hours longer in fresh formalin. They were dehydrated, cleaned in xylene, and mounted in paraffin blocks. Sections were always oriented at a right angle to the long axis of the left ventricle, and taken as close to the midpoint of the axis as possible.

MEASUREMENT OF SECTION THICKNESSES

Nuclear densities (number of nuclei per volume of tissue) were determined by measuring average section thicknesses. Using an ocular micrometer, the thicknesses of sections were determined by measuring the thickness of folds in the sections, i.e., parts of the section oriented at a right angle to the plane of the section. Sections without folds were not used for these determinations. Section thickness was considered to be satisfactorily determined when the standard error of the mean of the measurements was less than 2% of the mean. In most cases, about twenty measurements were required to satisfy this criterion. Sections without folds were not used for determination of nuclear densities. Fields to be studied were selected in a rigorously randomized manner.

PREPARATION OF NUCLEAR DNA

The excised heart was washed free of blood, ground with sea sand (Merck), and extracted with equal volumes of 6% 4-amino salicylate and phenol-cresol mixture (500 g phenol crystals, 70 ml redistilled cresol, 55 ml water, 0.5 g 8-hydroxyquinoline). DNA was precipitated with two volumes of ethanol, removed with a glass rod and washed with 75% ethanol. It was dissolved in standard sodium citrate and digested overnight at 37°C in a dialysis bag with RNase, 400 µg/ml, and pronase, 500 µg/ml, which had previously been heated to 80°C for 10 minutes at pH 5.0. The nuclear DNA was then separated from mitochondrial DNA after denaturation and renaturation by CsCl equilibrium centrifugation, precipitated by trichloroacetic acid, and its radioactivity was measured in a liquid scintillation counter as previously described (12, 13).

ESTIMATION OF ³H-THYMIDINE POOL SPECIFIC ACTIVITY

Excised hearts washed with saline were ground with sea sand and extracted with 5% trichloroacetic acid. The pyrimidine derivatives were separated from degradation products by charcoal absorption, and the radioactivity in the supernatant fluid was measured in a liquid scintillation counter as described by Zak et al. (12).

DETERMINATION OF HYDROXYPROLINE

Proteins containing hydroxyproline were meas-

ured in the residue remaining after digestion in 0.1N NaOH at 0°C. The residue was hydrolyzed in 6N HCl in a sealed tube for 20 hours at 110°C. Hydroxyproline was determined using *p*-dimethylamino-benzaldehyde by the method of Newman and Logan (14).

Results

INCORPORATION OF ³H-THYMIDINE INTO CARDIAC NUCLEAR DNA

Incorporation of ³H-thymidine into purified nuclear DNA was greatly increased after banding the aorta. This conforms with our previous observation that total cardiac DNA increased by 20% to 40%, following aortic constriction (1). In the first group the increase in heart weight was 8%, in the second 36% as compared with sham-operated litter mates. Fifty microcuries of ³H-thymidine per 100 g of body weight was injected on the second and third day after operation; the second injection was divided into three doses, 3 hours apart, in order to cover a longer period of the synthetic phase. Incorporation into nuclear DNA isolated from hearts enlarged by 8% was more than doubled, and incorporation into hearts enlarged by 36% was increased six times (Table 1).

SPECIFIC RADIOACTIVITY OF THYMIDINE POOL

To be sure that differences in incorporation of ³H-thymidine into DNA did not reflect changes in the specific activity of the precursor pool, the total radioactivity in trichloroacetic acid extracts of hearts and the radioactivity absorbed by charcoal (nucleosides and nucleotides) were measured in control hearts and in hearts after aortic constriction (Fig. 1). ³H-thymidine given intraperitoneally rapidly enters into heart muscle and reached a maximum concentration within 15 minutes after injection. ³H-thymidine was rapidly converted into noncyclic metabolites that are not absorbed by charcoal. The calculated half-life of the labeled thymidine pool was approximately 25 minutes. No difference in the pattern of accumulation or decay of radioactivity was found between hearts of control and banded animals. It is recognized that the immediate precursor in the synthesis of DNA, thymidine triphosphate, was not measured. However, the results indicate that

TABLE 1

Incorporation of ³H-Thymidine into the Nuclear DNA Isolated from Hearts of Sham-Operated and Banded Rats

Sham-operated counts/min/ μ g DNA	Banded	
	Increase in heart weight (%)	Counts/min/ μ g DNA
117	8	432
147	36	1152

³H-thymidine (50 μ c per 100 g body weight) was administered intraperitoneally to two groups of four animals on the second and third day after operation. Rats were killed 12 hours after the second injection.

the increased incorporation of thymidine into DNA is not due to changes in the specific activity of the precursor pool.

AUTORADIOGRAPHY

Two days after banding, hypertrophied hearts had a higher concentration of labeled nuclei than did sham-operated hearts. Sections from three hypertrophied and three sham-operated hearts were studied by autoradiography; the sections from hypertrophied hearts had an average of 33 labeled nuclei per square millimeter of area, while those from sham-operated hearts averaged 6.5 labeled nuclei per square millimeter of section. Thus nuclei incorporating tritiated thymidine were about five times more common in hypertrophied than in sham-operated hearts. Autoradiographs were developed at slightly varying times after the application of the emulsion, and meaningful comparison of grain counts in different specimens was therefore impossible. In one instance the left ventricle of a hypertrophied heart was compared to the right ventricle of the same heart in the same section. In this case the average number of grains per nucleus (12.0 in the right ventricle, 9.5 in the left) did not differ significantly ($P > 0.25$ by the Wilcoxon test [15]), and the rate of ³H-thymidine incorporation per labeled nucleus was not increased in the hypertrophied ventricle.

Labeling by ³H-thymidine was primarily confined to the nuclei outside muscle cells (Fig. 2, top). Only rarely was a labeled muscle nucleus found (Fig. 2, bottom). No labeled muscle nuclei were observed in the sham-operated hearts.

QUANTITATIVE HISTOLOGY

For each heart used, several sections were studied with random selection of fields. Nuclei were classified as of muscular, connective tissue, round cell, and unknown origin. In general, any nucleus that clearly lacked the morphology typical of the muscle cell, or was not surrounded by myofibril-containing eosinophilic material, was considered to be of connective tissue origin. "Round cells" were considered to be inflammatory cells and were classified separately. "Unknown" nuclei were

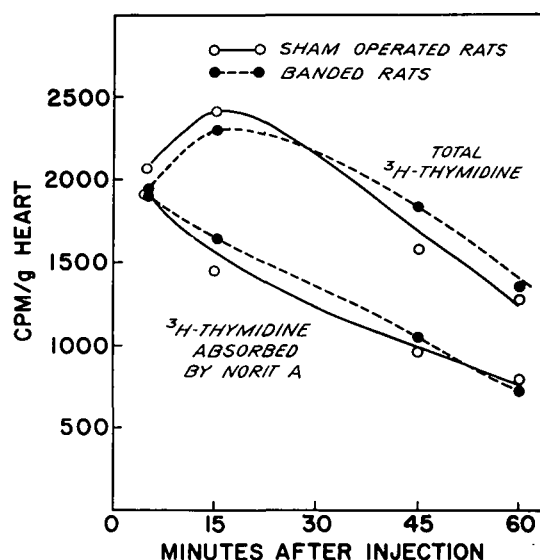
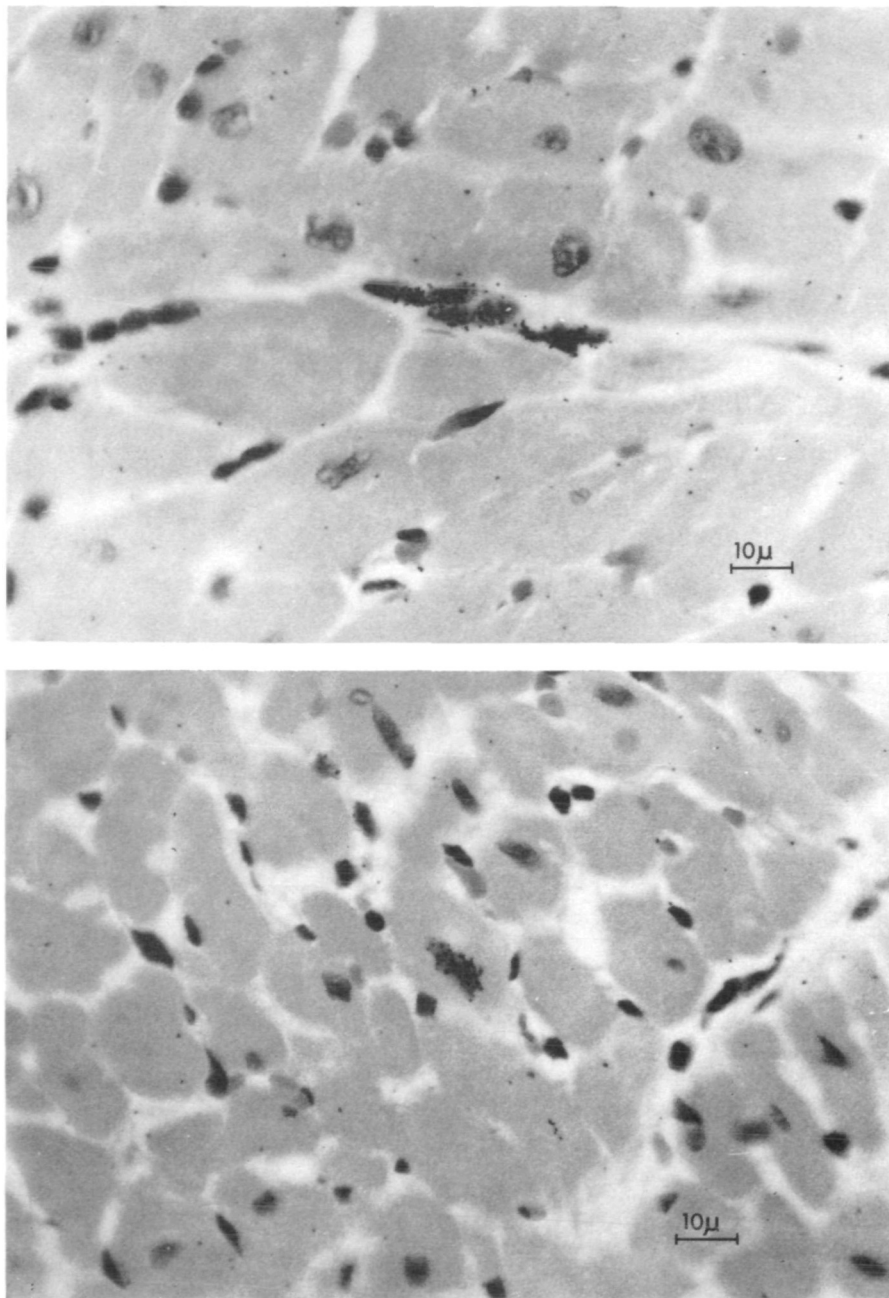


FIGURE 1

Radioactivity in trichloroacetic acid extracts of hearts from rats with aortic constriction and their sham-operated litter mates. (³H-thymidine absorbed by Norit A represents labeled cyclic thymidine derivatives.) Each point is an average of two determinations. (³H-thymidine, 5 μ c, was injected per 100 g body wt. 3 days after aortic constriction.

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**FIGURE 2**

Top: Several ^3H -thymidine-labeled connective tissue nuclei. Bottom: A muscle nucleus labeled with ^3H -thymidine (magnification 1,100 \times).

those that could not be classified with confidence.

In hypertrophied heart, muscle nuclei comprised a smaller proportion and connective

tissue nuclei comprised a larger proportion of the total nuclear population than in control hearts (Table 2). The muscle of control hearts at 12 to 13 or 17 to 18 weeks of age contained

TABLE 2

Frequencies of Types of Nuclei in Control and Banded Hearts

Animals	n		Muscle nuclei (%)	Connective tissue nuclei (%)	Round cells (%)	Unknown (%)
Controls, 12 to 13 weeks	4	Mean	25.3*	74.9§	0.3	0.5
		±SE	0.5	0.5		
Banded, 12 to 13 weeks	4	Mean	17.6†	82.0¶	0.3	0.2
		±SE	0.5	0.5		
Controls, 17 to 18 weeks	4	Mean	26.6‡	72.9**	0.2	0.3
		±SE	0.6	0.5		
Control, 6½ weeks	1		28.2	71.5	0.3	0.0

Comparisons by *t*-test: * vs. †, $P < 0.001$; † vs. ‡, $P < 0.001$; § vs. ¶, $P < 0.005$; ¶ vs. **, $P < 0.005$.

TABLE 3

Concentrations of Nuclei per Microliter of Tissue in Control and Banded Hearts

Group	n	Number of nuclei per microliter of tissue		
		Muscle nuclei	Connective tissue nuclei	All nuclei
Banded, 12 to 13 weeks	4	122,000	569,000	693,000
Control, 12 to 13 weeks	4	192,000*	564,000	762,000
Control, 17 to 18 weeks	4	198,000†	545,000	765,000
Control, 6½ weeks	1	291,000	735,000	1,030,000

*In comparison with the banded group, $P < 0.005$. †In comparison with the banded group, $P < 0.02$.

24% to 28% of the nuclei, whereas the muscle of hypertrophied hearts at 12 to 13 weeks of age contained only 17.6% of the nuclei. These differences are statistically significant. A younger (6½-week) control heart had virtually the same ratio of muscle to connective tissue nuclei as the older control hearts.

NUCLEAR DENSITIES

"Nuclear density" was defined as the number of nuclei per unit volume and was calculated from the formula: nuclei per unit volume of tissue = (number of nuclei observed in n fields) / ($n \times$ section thickness \times field area). In hypertrophied hearts the density of muscle nuclei was significantly reduced when compared with the densities of muscle nuclei in control hearts of the 12- to 13- and 17- to 18-week-old groups

(Tables 2 and 3). Neither the density of connective tissue nuclei nor that of the total nuclear population was significantly altered in hypertrophied hearts. In the single case studied, a 6½-week-old control heart had greater densities of both muscle and connective tissue nuclei than did any of the older hearts.

NUCLEI PER HEART

It was assumed that alterations in cardiac volume, with fixation, were identical in control and hypertrophic hearts and that such alterations were essentially negligible. On this basis, the numbers of the various types of nuclei per heart were calculated for each heart on the basis of the heart weight determined when the animals were killed and the assumption that in life the myocardial density is 1.10 (Table 4).

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TABLE 4

Number of Nuclei in Rat Left Ventricle

Group	n	Nuclei per heart $\times 10^{-8}$		
		Muscle nuclei	Connective tissue nuclei	All nuclei
Banded, 12 to 13 weeks	4	1.13	5.30	6.45
Control, 12 to 13 weeks	4	1.47*	4.28	5.81
Control, 17 to 18 weeks	4	1.71†	4.71	6.45
Control, 6½ weeks	1	1.81	4.54	6.35

*In comparison with the banded group, $P < 0.05$.†In comparison with the banded group, $P < 0.01$.

It follows from this calculation that the total number of muscle nuclei was not increased in the hypertrophied hearts, and may have been decreased, while the total number of connective tissue nuclei had apparently increased, especially in comparison with the control hearts of the same chronological age (12 to 13 weeks). Each nucleus was assumed to contain 6.2 pg DNA.

NUCLEAR DISTRIBUTION

It was of interest to determine whether the addition of connective tissue nuclei during hypertrophic growth was proportional to the number of such nuclei present at each point before banding, i.e., whether new connective tissue nuclei appeared in a uniform or a nonrandom distribution.

The hypothesis was made that the new growth was uniform. It was tested statistically by comparing, by the F-test (16), the variances about their means of the ratios (muscle nuclei/connective tissue nuclei) in individual microscopic fields of sections from hypertrophied and control hearts (see Appendix). Thus, instead of a comparison of these ratios themselves, comparison was made of their distributions in control and hypertrophic hearts.

If the F-test should demonstrate that the variance was significantly larger in hypertrophied than in control hearts, it would follow that connective tissue nuclei were less uniformly distributed after hypertrophy than

before. Conversely, a significantly smaller variance after hypertrophy would imply an even more uniform distribution of connective tissue nuclei than in controls.

If, in fact, such a test indicates that connective tissue nuclei have a less uniform distribution, it must follow that muscle cell nuclei are also more irregularly distributed. Since, however, no evidence was found for significant proliferation of muscle cell nuclei, any change in distribution was considered to be secondary to alteration in the growth pattern of connective tissue nuclei. Comparisons of these variances are presented in Table 5. The variance was significantly greater in hypertrophied hearts than in any group of control hearts considered separately, and also significantly greater than all controls considered as a single group. None of the control groups differed significantly from any of the other controls.

HYDROXYPROLINE ASSAYS

Hydroxyproline concentration in the left ventricle was initially decreased on the second day following aortic constriction but rose by the eleventh day to above control values (Table 6). The total hydroxyproline content ($\mu\text{g}/\text{heart}$) was initially unchanged from control values but also rose above control levels on the eleventh day.

Discussion

The data presented in this paper provide additional evidence that there is little or no

TABLE 5

Comparison by F-Test of the Distributions of Connective Tissue and Muscle Nuclei in Control and Banded Animals

Group	s ²	n		Group			
				Control 12-13 weeks	Control 17-18 weeks	Control 6½ weeks	All controls
Banded, 12 to 13 weeks	0.178639	183	F P	1.993 < 0.002	2.127 < 0.002	1.920 < 0.058	2.038 < 0.002
Control, 12 to 13 weeks	0.089624	116	F P		1.067 > 0.2	1.038 > 0.2	1.022 > 0.2
Control, 17 to 18 weeks	0.083968	121	F P			1.108 > 0.2	1.044 > 0.2
Control, 6½ weeks	0.093030	31	F P				1.061 > 0.2
All controls	0.087651	268					

The null hypothesis is that for any two groups compared $s_1^2 = s_2^2$. That the variance of the banded group is larger than that of any other group of hearts suggests that new connective tissue nuclei do not appear in strict proportion to the number of connective tissue nuclei already present at each point before banding.

TABLE 6

Hydroxyproline Content of Control and Banded Hearts

Group	n	Hydroxyproline (µg/100 mg wet weight)	Hydroxyproline (µg/heart)
Sham-operated, 2 days P (by one-tail t-test)	19	41.9 ± 1.3 < 0.01	267 ± 10 NS
Banded, 2 days	22	37.0 ± 1.3	274 ± 7
Sham-operated, 11 days P (by one-tail t-test)	8	47.0 ± 2.9 < 0.005	361 ± 21 < 0.005
Banded, 11 days	8	65.2 ± 4.2	603 ± 35

division of cardiac muscle cells following a stimulus to cardiac growth, although total cardiac DNA synthesis is markedly increased. Incorporation of tritiated thymidine into nuclear DNA was more than six times that of controls in hypertrophied hearts 2 days after banding. Although it is probable that mitochondrial DNA (17, 18) also is replicating at this stage, the separation of nuclear and mitochondrial DNA by CsCl gradient centrifugation eliminates the contribution of extranuclear DNA. The size of the thymidine pool as estimated above apparently did not change during the experimental procedure, therefore

changes in the size of the pool of thymidine triphosphate probably did not account for the increased thymidine incorporation in the hypertrophied hearts.

Radioautography clearly showed that the incorporated tritiated thymidine was almost exclusively associated with nuclei of the connective tissue. The rare muscle nucleus that contained radioactivity may possibly represent a polyploid cell.

Additional evidence that there was no significant proliferation of muscle cells comes from the quantitative determination of cell types. Muscle nuclei made up a smaller

fraction of the total number of nuclei in the hypertrophied hearts. The decreased proportion of muscle nuclei was probably secondary to both a decrease in number of muscle nuclei and to an increase in the number of connective tissue nuclei. The decrease in absolute number of muscle nuclei in hypertrophy was statistically significant. Although the observed increase in number of connective tissue nuclei was not statistically significant, the increase is likely to be real. The significant increase in total ventricular DNA content, coupled with the increase in ratio of connective tissue to muscle nuclei, clearly demonstrates that an increase in total number of connective tissue cells must have occurred.

Thus, in our system, proliferation of muscle cells during hypertrophic growth lags far behind the proliferation of connective tissue nuclei and probably occurs to a very limited extent, if at all. Similarly, in the diaphragm (12) increases in DNA content with hypertrophy are also associated with proliferation of connective tissue elements. These results will be discussed with reference to (1) ability of muscle cells to multiply; (2) connective tissue hyperplasia; (3) relevance to other types of cardiac hypertrophy.

ABILITY OF MUSCLE CELLS TO MULTIPLY

The cardiac muscle cell apparently loses its ability to divide fairly early in its life. Not all authors are agreed on the exact time at which this ability is lost, and more particularly there is lack of agreement concerning the morphologic and biochemical conditions in the cell which necessitate the end of mitotic activity. Skeletal and cardiac muscle may not be exactly alike in this respect. Stockdale and Holtzer (19) have shown that in developing skeletal muscle DNA synthesis and mitotic cell divisions are incompatible with contractile activity; once the dividing mononucleated myoblasts fuse to form multinucleated myotubes DNA synthesis ceased in the myotubular nuclei. The results concerning the differentiating heart are less conclusive. In some studies no mitotic figures were reported in cells containing myofibrils (20-22). Recently, Manasek has shown (23) by electron micro-

scopy that both contractile and undifferentiated cells are able to divide, confirming the previous light microscopic studies of Mark and Strasser (24) and DeHaan (25). Thus the differentiation of the heart muscle seems to have features somewhat different from those of skeletal muscle, although these differences may not be basic. As Ishikawa and associates (26) state: "in skeletal muscle the decision to synthesize contractile proteins is coupled with the decision to withdraw from the mitotic cycle. In cardiac muscle it may take another division or two before the decision to withdraw from the mitotic cycle takes effect."

It is generally agreed that the number of mitoses in the heart decreases rapidly after birth (27-32). Although the nuclear density in the growing heart decreases, the total number of nuclei in adult mammalian heart muscle is twice the early postnatal value (33). Hort (33) supports the thesis that amitotic divisions are the source of new nuclei, but a recent electron microscopic study of the postnatal growing heart by Shafiq et al. (34) reports that colchicine arrests mitoses in undifferentiated myoblasts and other "free" cells, but not in the already formed muscle fibers. The presence of undifferentiated presumptive muscle cells in developing heart was also described by Wainrach and Sotello (20). Although the abundant satellite cells in developing skeletal muscle may possibly be myogenic cells (e.g., 34), such subsarcolemmic satellite cells are not found in developing heart muscle.

In view of the progressive decline in mitotic rates and nuclear densities with age, heart muscle might be considered biochemically adult when DNA synthesis effectively stops. In rats 48 days after birth no more DNA synthesis could be detected (28). In mice 5 weeks after birth fewer than 0.1% of the nuclei are labeled and those are of endocardial, endothelial, and connective tissue (29).

Although DNA synthesis in the adult heart is at very low levels, some incorporation of labeled thymidine into DNA is always measurable both chemically (4, this paper) and by radioautography (7, 32). The number of

labeled nuclei exceeds the number of observed mitoses by a factor of 10 (7, 32). This discrepancy is interpreted by Pelc (7) as evidence for a small amount of "metabolic" DNA, while Klinge (32) explains this observation by "collapsed" mitoses which do not lead to cytoplasmic divisions.

Evidence presented in this paper and by others (2-4) indicates that DNA synthesis is not resumed in differentiated heart muscle cells in the case of cardiac hypertrophy, a situation similar to that of skeletal muscle (e.g., 12, 18). Reports of DNA synthesis in muscle cells of hypertrophying left ventricles of banded rats (35) and in atria (but not ventricles) of hearts with ventricular infarction (36) stand in contrast to the above finding. Improved techniques such as thin-section radioautography or evaluation with the electron microscope will be necessary to make the identification of cell types less ambiguous and results more conclusive.

CONNECTIVE TISSUE HYPERPLASIA

In contrast to the apparent inactivity of the muscle nuclei during hypertrophy, the connective tissue nuclei become quite active, both in terms of mitotic activity and uptake of tritiated thymidine. Increased proliferation of connective tissue nuclei is associated with increased synthesis of collagen. Enhanced synthesis of DNA was noticed on the second day after banding and reached a peak on the seventh day (3, 4). No change was detected in the collagen content of the myocardium on the second day, but by the eleventh postoperative day it had increased by approximately 67%. Thus the new connective tissue nuclei and collagen are produced at about the same time. Increased collagen content has been shown to accompany cardiac enlargement produced by other methods: narrowing of the aorta in rats (37) and pulmonary artery in cats (38), administration of isoprenaline, long-term adaptation to hypoxia, and physical stress (37). Exceptions were cardiomegaly produced by anemia, where collagen content did not change, and the effects of thyroxine administration, where the collagen content increased only moderately (37).

As to the location of collagen production, no attempt was made to distinguish between the synthesis of collagen in connective tissue of the interstitium and in the blood vessels. There are different opinions about changes in the number of capillaries in the hypertrophying heart. No change was reported by Roberts and Wearn (39), while Linzbach (40) found that the ratio of capillaries to the cross-sectional area of the muscle fibers is relatively constant during hypertrophy except in the advanced stages. The thickness of the blood vessel wall is said to increase (41).

A statistical analysis of the spatial distribution of connective tissue nuclei demonstrated that the distribution was different after hypertrophy. If muscle cell nuclei are considered as a fixed matrix in space, then connective tissue nuclei are arranged more irregularly in this matrix after hypertrophy than before it. Alternatively, new connective tissue nuclei are not produced in proportion to the number already present at each point before hypertrophic growth starts.

Our observations are consistent with the hypothesis that acute constriction of the aorta may lead to focal or scattered necrosis with subsequent replacement by connective tissue.

RELEVANCE TO OTHER TYPES OF CARDIAC HYPERTROPHY

The observations made in this paper refer to cardiac hypertrophy that is produced rapidly. It is not known whether the generalization that division of cardiac muscle cells does not occur in cardiac hypertrophy in the adult applies to all pathological or experimental situations. To date, however, in contrast to skeletal muscle, no evidence for cardiac muscle regeneration has ever been obtained. It is likely that under ordinary physiological and pathological situations it does not occur. That does not necessarily mean that the potential for muscle cell proliferation has been lost but only that it is not stimulated under the pathological and experimental conditions so far examined.

Clinical cardiac hypertrophy is likely to have a more gradual onset than is produced by experimental aortic constriction. The

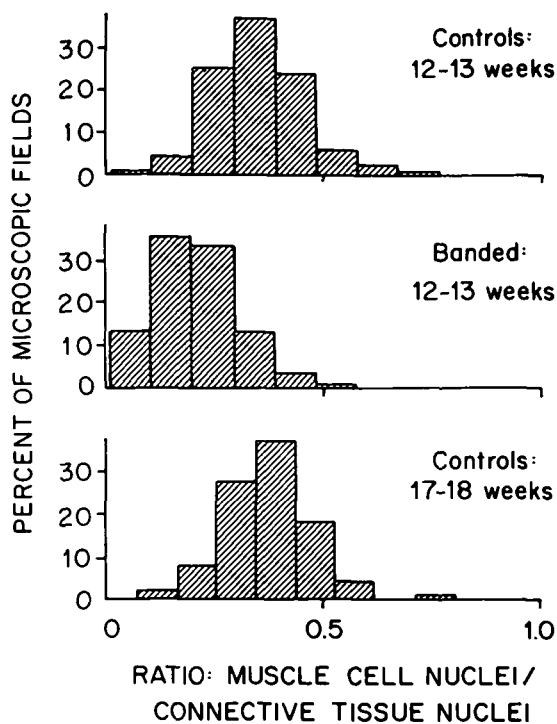


FIGURE 3

The ratio of muscle nuclei to connective tissue nuclei in control and hypertrophied hearts.

abrupt hyperplasia of connective tissue cells observed in this study may be related to the rapid appearance of hypertrophy, and may be modified in other types of more slowly developing cardiac enlargement.

The cardiac hypertrophy occurring in congenital lesions in the young may differ from the model studied here. The potential for muscle cell division is retained in embryonic and young muscle cells, and it may be that stimuli for cardiac growth lead to cell division under these circumstances. More work must be done to clarify this point.

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Appendix

To test whether changes in the distribution of connective tissue nuclei, relative to the

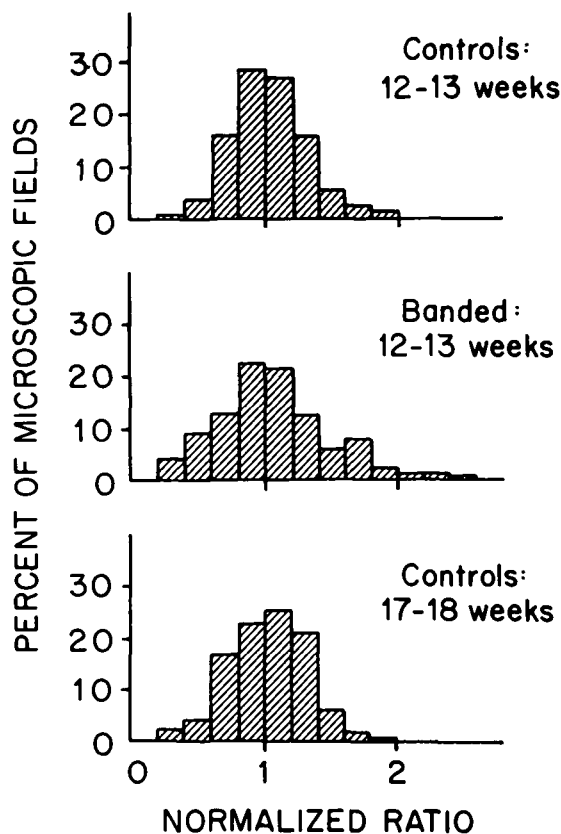


FIGURE 4

Plot of the value of the "normalized" variable, r_i/R , in control and hypertrophic hearts. The distribution of the variable r_i/R in banded animals is not significantly different from the normal ($P > 0.05$ by χ^2).

distribution of muscle cell nuclei, had occurred, it was necessary to devise a variable whose variance would not be greatly affected by the change in the ratio of muscle to connective tissue nuclei that was found with hypertrophy. This was accomplished as follows: Given a section of tissue in which P total nuclei including M muscle nuclei and C connective tissue nuclei, are counted in N total fields, $M + C = P$. If in each field scored, m_i ($i = 1, 2, \dots, N$) muscle and c_i connective tissue nuclei are counted,

$$\sum_{i=1}^N m_i + \sum_{i=1}^N c_i = M + C = P.$$

Define the average ratio of muscle to connec-

tive tissue nuclei for the section (or heart), $R = M/C$. For each field $r_i = m_i/c_i$, and for a large number of fields

$$\left(\frac{\sum_{i=1}^N r_i}{N} \right) / N = \bar{r}_i \approx R, \text{ and } \bar{r}_i/R \approx 1.$$

One may call r_i/R a "normalized" variable. It is seen that r_i/R has an average value close to 1, regardless of the average value M/C , and that the variance of

$$r_i/R, \quad s^2_{r_i/R} = \sum_{i=1}^N \left[\frac{(r_i/R) - \left(\frac{\sum_{i=1}^N r_i/NR}{N-1} \right)}{N-1} \right]^2$$

provides a means of comparing the shapes of the distributions of r_i/R in different sections or different hearts. In the material studied in these experiments, typical values for R were 0.35 for control hearts, and 0.21 for hypertrophic hearts. In Figure 3 are plotted the values of m_i/c_i for microscope fields in sections from control and hypertrophic hearts, and in Figure 4 are plotted the values r_i/R for the same hearts. Clearly, the variance $s^2_{r_i/R}$ is larger in the case of the hypertrophic hearts. This change in variance with hypertrophy is detected by the F-test, and suggests a non-uniform growth of connective tissue.

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