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ORIGINAL ARTICLE

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DNA content and expression of cell cycle proteins in caterpillar nuclei from fetal human cardiac myocytes

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Abstract Caterpillar nuclei (CN) are characterized by their peculiar morphology, with chromatin distributed in clusters and running along the longitudinal axis of the nucleus. They can be observed in normal hearts of fetuses as well as in hearts of children and adults with rheumatic heart disease. This study has demonstrated by means of ploidy studies with digital image analysis that in the fetal heart (20.5±1.8 weeks) the CN (diploid = 5.6±8.4%; tetraploid = 46.2±24.2%; hypertetraploid = 46.9±26.3%) present higher DNA content than non-caterpillar myocyte nuclei (diploid = 89.4±6.2%; tetraploid = 10.0±4.1%; hypertetraploid = 1.5±1.3%) (P=0.000001, 0.00013, and 0.000038, respectively). Expression of proliferation cell nuclear antigen (PCNA; 30.6±11.7% in CN and 13.4±7.3% in non-caterpillar myocyte nuclei; P=0.0115) and cyclin B1 (2.8±3.8% and 12.6±15.6%, respectively; P=n.s.) was also positive in these nuclei. In conclusion, these results suggest that there exists a relationship between CN morphology and myocyte replication phenomena.

Keywords Caterpillar nuclei · Cyclin B1 · Fetal heart · Nuclear ploidy · PCNA

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Introduction

At the beginning of the century Von Oppel reported for the first time the presence of caterpillar nuclei (CN) in the rabbit myocardium as a reaction to implantation of a needle [19]. He observed that the muscle fibers in the puncture canal showed hypertrophic nuclei with the chromatin distributed in granules or clusters with a "serrated stripe" or condensed in a "single thread running in the long axis" of the nucleus. Anitschkow made a similar observation some years later and focused on the peculiar chromatin condensation of such muscle fibers, which came to be known as "Anitschkow cells or myocytes", characteristic of the Aschoff bodies in rheumatic heart disease [1].

Anitschkow cells and CN were popular subjects in the literature of the first half of the last century, but these are topics seldom discussed in the past 50 years. The origin and significance of the heart CN have not been established, but on account of their presence in the fetal life, when the heart growth is due to hyperplasia, it has been speculated that their presence may be related to myocyte replication phenomena [14]. In order to find out whether CN present evidence of DNA replication, we decided to study the ploidy status and activity of proliferation cell nuclear antigen (PCNA; a protein expressed during late G1, S, G2 and M phases of the cell cycle) and cyclin B1 in CN and compare them with those in the non-caterpillar myocyte nuclei.

Materials and methods

Tissue fixation and sampling

For the present study, we employed the hearts of ten human fetuses with minimal or no maceration, gestational age 17–23 weeks, established by the predicted menstrual age, Sheppard's tables for fetal weight [5] and by fetal foot length [12]. This gestational age was chosen because of the high proliferative index of the heart during that time. As controls, we employed four hearts collected from a full-term newborn and 1-, 5- and 7-month-old babies. Hearts showed no evidence of myocardial infection.

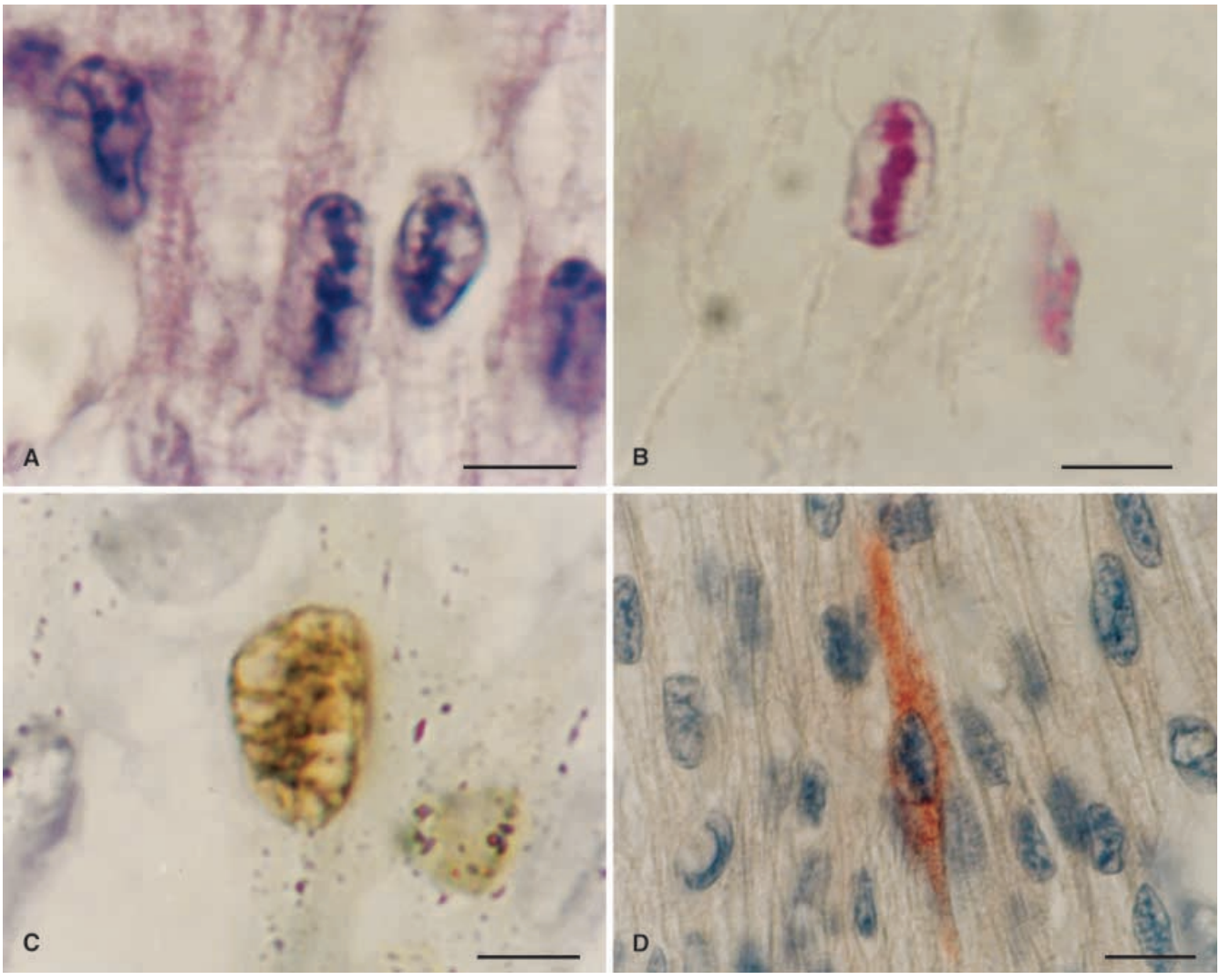

Fig. 1 Morphology of myocytes with caterpillar nuclei (CN): **A** hematoxylin-eosin and **B** Feulgen stain of CN showing the central condensation of chromatin (×1000; *bar* 5 µm); **C** proliferation cell nuclear antigen (PCNA) stain of a CN (diaminobenzidine, ×1000; *bar* 5 µm); **D** the presence of cyclin B1 can be observed in the cytoplasm of a fetal myocyte with CN [aminoethyl carbazole (AEC), ×400; *bar* 10 µm]

Gestational age, sex, weight, fetal dimensions and clinical data are summarized in Table 1. The hearts were removed, sectioned in halves, fixed in 10% buffered formalin and embedded in paraffin. Several 3-µm thick tissue sections from the left ventricular free wall and interventricular septum were stained with hematoxylin-eosin and Feulgen's (Fig. 1a, b) and for immunohistochemical studies.

This study was performed in accordance with the ethics standards of the 1964 Declaration of Helsinki. Parents gave their informed consent prior to the inclusion in the study.

Quantitative analysis of the incidence of CN in myocardial cells

The frequency of CN in myocytes was established in tissue sections stained with hematoxylin-eosin on 40 microscopic fields examined at a 400× magnification.

Determination of DNA content

Nuclear DNA content of individual nuclei was determined by means of densitometry with a digital analysis system (Vidas-Kontron-Zeiss, Germany) with DNA Vidas software in the Feulgen stained-tissue sections. This method is based on the interaction between Schiff's reagent and the aldehyde groups of the deoxyribose molecules, previously unmasked by acid hydrolysis. In order to maximize nuclear detail, the staining process was optimized at 55 min [10]. For evaluation of DNA content by static cytomorphometry, myocytes transversally sectioned were measured in 3-µm tissue sections; only cells containing a centrally located nucleus, longitudinally oriented, and located equidistant to the cell boundaries were analyzed. Nuclei that appeared to be overlapping or not clearly defined were excluded from the study [7, 13].

The ploidy status of 300 non-caterpillar myocytes and 40–60 CN for each case was determined using the interstitial cells as diploid reference cells. In addition, the DNA content of the CN was studied employing the DNA content of non-caterpillar myocyte nuclei content as the baseline 2c reference value.

Determination of PCNA and cyclin B1

Immunohistochemical staining was performed using mouse monoclonal antibodies to PCNA (clone PC10, 1:50; Dakopatts, Denmark) and cyclin B1 (clone 7A9, 1:30; Novocastra, UK). The de-

Table 1 Summary of fetal dimensions and clinical data. Age is expressed as gestational age in weeks

Case	Age (weeks)	Sex	Weight (g)	Crown–rump (cm)	Crown–heel (cm)	Foot length (mm)	Pathology	Manner of delivery
1	17	Female	235	14.1	21.5	26.3	Anencephaly	Vaginal
2	18	Male	220	13.5	21.1	25.1	Deciduitis	Vaginal
3	21	Male	490	17.2	25.2	33.5	Holoprosencephaly	Vaginal
4	21	Male	510	16.8	25.3	34	Anencephaly	Vaginal
5	21	Male	245	14.2	20.1	32.5	Anencephaly	Vaginal
6	21	Male	515	20.4	30	37.3	Chorioamnionitis	Vaginal
7	21	Male	400	18.2	26.2	35	Chorioamnionitis	Vaginal
8	21	Female	280	15.3	21.1	32.6	Chorioamnionitis	Vaginal
9	23	Female	570	16.2	26.5	43.9	Chorioamnionitis	Vaginal
10	21	Female	365	13.6	20.8	37.1	Chorioamnionitis	Vaginal

Table 2 Incidence, ploidy status and proliferation cell nuclear antigen (PCNA) activity of caterpillar nuclei (CN). Age is expressed as gestational age in weeks for stillborns and days or months for liveborns. *%CN* CN per 1000, *Ploidy* DNA content, *2c* percentage of diploid nuclei, *4c* percentage of tetraploid nuclei, *>4c* percentage of hypertetraploid nuclei, *PCNA* expression of PCNA as a percentage in non-caterpillar myocytes, *PCNA CN* expression of PCNA as a percentage in myocytes with CN

Case	Age	% CN	Ploidy			Ploidy (CN)			PCNA	PCNA (CN)
			2c	4c	>4c	2c	4c>	4c		
1	17 weeks	0.5	98.2	1.4	0.4	0	0	100	10.3	13.4
2	18 weeks	6.6	80.3	14	5.7	10	10	80	19.5	45.4
3	21 weeks	0.4	95.2	4.2	0.3	0	56	44	25.6	33.9
4	21 weeks	1.1	85.1	13	1.9	21.5	44	34.5	21.5	44.4
5	21 weeks	0.4	94.7	5.2	0.1	0	57	43	1.68	46.1
6	21 weeks	1.5	89.1	9.1	1.8	0	61.2	38.8	10.6	30
7	21 weeks	5.6	93.1	4.8	2.1	6.9	72.1	21	4.5	16.6
8	21 weeks	6.7	85.4	11.2	3.4	13.9	45	41.1	13.7	28.
9	23 weeks	1	90.7	8.8	0.5	0	44.4	55.6	12.9	24
10	21 weeks	2.7	85.5	14.1	0.8	16.6	72.4	11	13.9	24.2
Mean	20.5	2.7	89.4	10.05	1.56	5.68	46.2	46.9	13.4	30,6
SD	1.81	2.6	6.28	4.17	1.37	8.43	24.2	26.3	7.3	11.7
11	1 day	0.2	85.59	13.56	0.85				3.1	
12	1 month	0	92.04	7.96	0				2	
13	5 months	0	99.2	0.8	0				0.9	
14	7 months	0	96.2	2.9	0.9				1.2	

tection was performed with the immunoperoxidase technique based on a biotin–streptavidin system (Multilink Biogenex, San Ramon, Calif.). The proportion of positively stained caterpillar and non-caterpillar myocyte nuclei was determined in the left ventricle and interventricular septum at ×400 for each case.

Desmin, α-sarcomeric actin and α-smooth muscle specific actin staining

To demonstrate that PCNA and cyclin B1 labeling and caterpillar morphology occurred in myocyte nuclei, sections from a limited number of samples were stained with monoclonal antibodies to desmin (1:100; Biogenex, Calif.), α-sarcomeric actin (alpha-Sr-1, 1:30; Dakopatts, Denmark) and α-smooth muscle specific actin (1A4, 1:40; Dakopatts). The detection was performed with the immunoperoxidase technique (Multilink Biogenex) in conformity with the manufacturer's recommendations.

Statistical analysis

Measurements are presented as mean ± SD. Significance for comparisons among groups was determined using an analysis of variance (ANOVA) method. Analyses were performed using Statistica for Windows (release 4.2) statistical software. Values of $P<0.05$ were considered to be significant.

Results

Proportion of CN

CN were identified in the hematoxylin-eosin and Feulgen-stained histologic sections by their peculiar morphology (Fig. 1a, b). In the 17- to 23-week-old fetuses, the proportion of CN was variable, ranging from 4 to 67 for 1000 non-caterpillar nuclei. In the newborn and infant hearts, the presence of CN was exceptional (Table 2).

Demonstration of the muscular origin of caterpillar nuclei

Samples were stained with the following antibodies: anti-desmin antibody, an intermediate filament [15] expressed in cardiac, skeletal and smooth muscle; anti-α-sarcomeric actin, a sarcomere-specific protein [9], and anti-α smooth-muscle-specific actin (negative in myocytes with CN). CN were present in cells expressing α-sarcomeric actin and desmin.

Ploidy status of non-caterpillar myocyte nuclei and CN

Table 1 shows that most of the non-caterpillar myocyte nuclei in the 17- to 23-week-old fetuses were 2c, with a small proportion of 4c, probably corresponding to cells in the G2/M stage of the cell cycle. With the exception of one case, the proportion of hypertetraploid nuclei was very low (case 2).

In the newborn and infant hearts, the proportion of 2c nuclei was even larger than in the younger fetuses and almost no tetraploid nuclei were observed (Table 2). In the fetal hearts, over 94% of CN had a DNA content higher than 2c, half of them showed tetraploid values and the remaining half were hypertetraploid (Table 2).

The difference between the ploidy status of the myocyte nuclei with conventional morphology and the CN was statistically significant (2c P=0.000001; 4c P=0.00013; >4c P=0.000038).

Expression of PCNA and cyclin B1

PCNA labeling was observed in 13.4±7.3% (mean±SD) of non-caterpillar myocyte nuclei. There was no difference between the left ventricular wall and the interventricular septum. Myocytes with CN morphology showed 30.6±11.7% of PCNA-stained nuclei (P=0.00096; Fig. 1c).

The expression of a G2/M marker, cyclin B1, showed a cytoplasmic stain in 12.6±15.6% of myocytes. In myocytes with CN, 2.8±3.8% were positive (P=n.s.) for cyclin B1 (Fig. 1d).

Discussion

The peculiar morphology of CN can be observed in normal hearts of fetuses and to a lower extent in full-term infants [18] as well as in hearts of children and adults with rheumatic heart disease [16, 17]. Most studies on the origin of CN were carried out in the pre-immunohistochemical era and their myocytic origin was controversial. On the basis of cell size, shape and presence of cross striations, most reports agreed that CN belong mainly to myocytes. The biological meaning of CN in myocytes has not been established.

The presence of CN in normal hearts with high proliferative activity and in young patients with cardiac rheumatic disease favors the idea that CN may be related to myocyte proliferation. This possibility led us to study the expression of some proteins (PCNA, cyclin B1) essential for controlling cell-cycle progression in myocytes with CN.

The PCNA protein expression is a useful immunohistochemical method for determining the proliferative activity of cell populations, although there exist certain restrictions on its interpretation since it is not a cellular cycle-specific marker: PCNA expresses itself in replicative cells and in those with DNA repair processes [2]. The high percentage of caterpillar nuclei (30%) expressing PCNA indicates that this peculiar morphology is related to an increase in DNA synthesis and proliferative activity.

The B1 protein affects the G2 to M transition and it is, therefore, restricted to a specific period of the cell cycle. The type-B cyclins associate with Cdk1 and these complexes are the mitosis-specific kinases [4, 11]. The high level of expression in cytoplasm (and in a lesser extent in nuclei) of fetal myocytes and in a percentage of CN demonstrate that these cells are in an active process of proliferation.

The ploidy studies of CN presented largely tetraploid and hypertetraploid values, which indicate intense DNA synthesis and probably a marginal polyploidization phenomenon in the fetal heart. DNA hyperploidy values have been communicated in previous studies with a low rate of normal myocyte nuclei [20].

CN can also be a morphologic expression of an alternative mechanism for the formation of polyploid nuclei, which can include endomitosis or endoreduplication [8]. Endoreduplication represents a cell cycle that omits the mitotic phase; whereas, in endomitosis, mitosis takes place within the nuclear envelope without the formation of a mitotic spindle. The central localization of the chromatin thread in the largest diameter of the nucleus can also be suggestive of an intranuclear metaphasic plate, as is the case in other eukaryotic cells [21]. In Fig. 1c, a PCNA-stained CN shows a pattern characteristic of chromosomes arranged in a metaphasic plate. Other authors suggested the possibility that CN represent a postmitotic chromatin organization [3] or a morphological expression of a response to hypoxic cardiac injury [6]. Irrespective of these hypothetical interpretations, our results, showing that CN contain an increased amount of DNA and express PCNA, a regulator of the cell cycle, support the opinion that this peculiar morphological change is related to myocyte replicative mechanisms.

It is very difficult to provide evidence of the exact position of these cells in the cell cycle in tissues obtained from human necropsies. On the basis of the expression of PCNA in a large proportion of CN, the occasional positivity of cyclin B1 and the high ploidy status, we can speculate that CN may belong to cells undergoing endomitosis. Further studies using fresh tissues would be required to determine whether the cell cycle proteins are involved in the genesis of CN.

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