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Developmental expression of troponin I isoforms in fetal human heart

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We have used antibodies specific for troponin I proteins to examine human cardiac development and have detected a transiently expressed developmental isoform. This isoform is distinct from adult cardiac troponin I (TnIc) but is indistinguishable, on the basis of electrophoretic mobility and antibody reactivity, from the isoform found in slow skeletal muscle (TnIs). Furthermore, we show that mRNA for TnIs is present in fetal, but not adult, heart. Analysis of a developmental series of fetal samples indicates that there is a transition in expression from TnIs to TnIc which occurs between 20 weeks fetal and 9 months postnatal development.

Troponin I; Cardiac development

1. INTRODUCTION

The troponin complex forms a calcium-sensitive switch on the thin filament of striated muscle which is involved in regulating muscle contraction. It is comprised of three proteins: troponin I (TnI), troponin T (TnT) and troponin C (TnC). Multiple isoforms of each of these proteins exist and are expressed in a tissue-specific and developmentally regulated manner. For troponin I, three principal isoforms have been described on the basis of protein sequencing [1,2], antibody studies [3–7] and, more recently, by molecular cloning [8–13]. These isoforms are associated with fast skeletal muscle, slow skeletal muscle or cardiac muscle, and are referred to here as fast TnI (TnIf), slow TnI (TnIs) and cardiac TnI (TnIc), respectively.

The cardiac isoform differs from those found in skeletal muscle by the presence of an extended N-terminal sequence. This sequence contains two adjacent serine residues which are phosphorylated in response to β-adrenergic stimulation in the heart [14–16] as part of the phosphorylation-mediated control of cardiac contraction by adrenergic agonists. This is an important feature of the cardiac isoform and the amino acid sequence of the phosphorylation site is conserved between all species so far examined including that of man [8,16].

In rat and chick, a developmental isoform of troponin I has been described in fetal heart which is indistinguishable from that present in slow skeletal muscle [5,6,17]. In rat, this is the only isoform found during early cardiac development, and there is a transition to expression of TnIc during the perinatal period [5,6]. Moreover, molecular cloning has shown that this is associated with a transition in the accumulation of mRNAs encoding TnIs and TnIc [13]. In chick the situation is different as both TnIs and TnIc are expressed at all stages of cardiac development examined in ovo, with TnIs being lost post-hatching [6]. In man, few studies have been carried out on troponin expression during cardiac development, and no developmental isoforms of troponin I have been described in the fetal heart (e.g. [18]).

Here we describe the presence of a developmental isoform of troponin I in the human fetal heart, and demonstrate that this isoform corresponds to that found in slow skeletal muscle.

Abbreviations: TnI, troponin I (TnIc, TnIf and TnIs; cardiac, fast skeletal and slow skeletal muscle isoforms, respectively).

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2. MATERIALS AND METHODS

2.1. *Antibodies used for troponin I detection*

Monoclonal antibodies used for the detection of human troponin I isoforms were derived from previously described clones [19]. Antibody derived from clone 42/25 specifically reacts with all three troponin I isoforms in chicken and rat [6]. Sheep polyclonal antibody was prepared using troponin I isolated from human heart and has been previously shown to be specific for this isoform in both man and rat [7].

2.2. *Preparation of protein extracts and immunoblotting*

Cardiac muscle was obtained from elective abortions, from autopsy and from samples taken at the time of surgery and stored in liquid nitrogen prior to use. Age of fetal samples was determined by foot measurement and quoted figures refer to gestational age. All samples were judged to be from non-pathological tissue. Proteins were pre-

pared by pulverising tissue while frozen, followed by homogenising in 1% SDS. Protein concentrations were determined [20] and 50 μg samples were run on 10% polyacrylamide gels containing 1% SDS [21]. Following electrophoresis, gels were equilibrated in 20 mM Tris base/150 mM glycine and electroblotted onto nitrocellulose filters. Western blots were stained with antibody diluted 1/3000 (42/25) or 1/10 000 (cardiac-specific) in phosphate-buffered saline containing 0.1 % Tween 20. Peroxidase-linked rabbit antibodies to mouse or sheep immunoglobulins (Dakopatts) were used to localize primary antibody binding [6].

2.3. *RNA preparation and Northern blot analysis*

RNA was prepared from cardiac and skeletal muscle samples using guanidium isothiocyanate and CsCl centrifugation [22]. Northern blots were prepared using 20 μg of total RNA run on 1.5% agarose-formaldehyde gels [23] and transferred to Hybond N+ membranes (Amersham UK) according to manufacturers specifications. A cDNA fragment containing 3′ non-coding sequence of human TnIs mRNA was generated by polymerase chain reaction as follows: 50 pmol of each of two primers (5′-GCTCTGAGCCTCCTGCTT-3′ and 5′-GTTTAATCCAGTTCCA-3′) were added to 500 ng human genomic DNA, 100 mM each of dATP, dCTP, dTTP and dGTP in 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 600 μM $MgCl_2$, 100 μg/ml bovine serum albumin, and annealed by heating to 94°C for 5 min and cooling to 55°C. Taq polymerase (NBL) was added and 30 cycles carried out at 72°C (2 min), 94°C (1 min) and 55°C (2 min). The resulting 136 bp DNA fragment was isolated by separating the PCR products on Nusieve agarose (FMC Bioproducts) and cutting out the appropriate band. ^{32}P-labelled probes were prepared from the TnIs cDNA fragment or from the insert of clone pCTI-2 encoding TnIc [8] by priming using standard conditions [23].

3. RESULTS

3.1. *Detection of troponin isoforms present in the human heart*

Fig. 1A shows total protein extracts from adult and fetal heart analysed on Western blots using previously characterised monoclonal antibodies. Antibody 42/25 has previously been shown to detect all three isoforms of troponin I (TnIf, TnIs and TnIc) in chicken and rat [6], or data demonstrate that this is also true for man. In adult cardiac (atrial) muscle a single protein band is detected corresponding to TnIc, whereas in fetal skeletal muscle, which contains a mixed population of fast and slow muscle fibres, two protein bands are detected corresponding to TnIf and TnIs. The relative position of human TnIs and TnIf was determined by comparison with published data for human troponin isoforms (e.g. [3]).

In fetal heart at 8 weeks, a single predominant band is seen which is clearly different from TnIc, but which is indistinguishable from that of TnIs on the basis of its electrophoretic mobility. In the 33-week fetal heart sample the same band is seen together with TnIc. This band is not seen in the adult atrial sample. Fig. 1B shows a parallel filter hybridized with antibody specific for cardiac troponin I [7], and no bands are seen in the human fetal skeletal muscle track demonstrating the specificity of detection using this antibody. In the 8-week fetal heart sample trace amounts of TnIc are visible using this antibody, but the predominant troponin protein

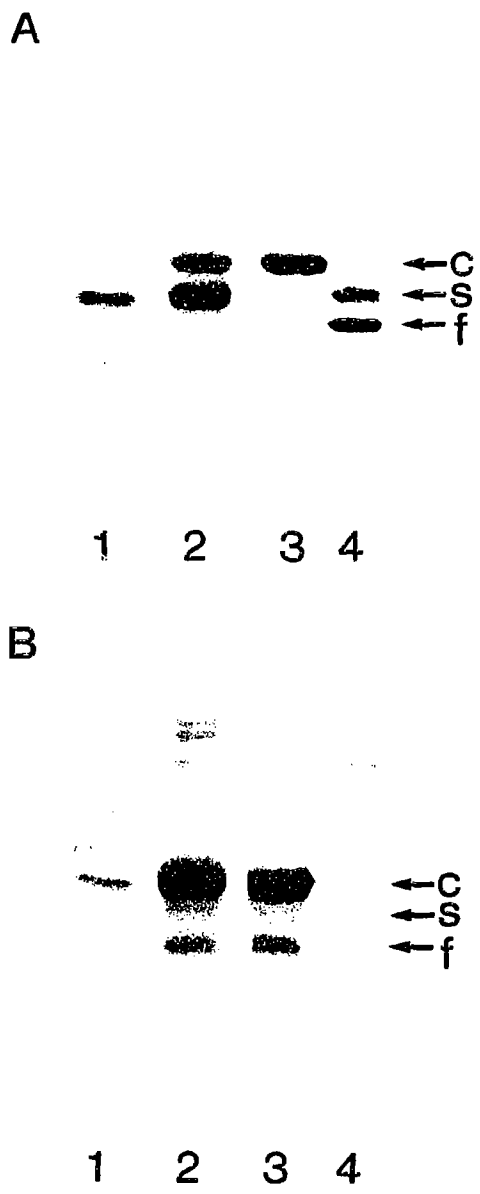

Fig. 1. Detection of troponin I isoforms in human heart. Parallel immunoblots of protein extracts from 8-week fetal heart (lane 1), 33-week fetal heart (lane 2), adult atrial muscle (lane 3) and fetal skeletal muscle (lane 4). (A) Detection of troponin isoforms with antibody 42/25 which detects all three isoforms TnIc (C), TnIs (S) and TnIf (F). (B) Detection of parallel samples with sheep polyclonal antibody specific for cardiac troponin I.

detected with antibody 42/25 in Fig. 1A, is not detected. In the 33-week fetal heart and adult atrial samples the major band seen in TnIc. A second, minor band which runs at a similar position to TnIf is also detected in these samples. This is almost certainly a breakdown product of TnIc. Firstly, because it is detected with the cardiac-specific antibody whereas TnIf is not and secondly, because it is more evident in samples where protein degradation is apparent (data not shown) as has been previously described [3]. Together, these data suggest that the slow skeletal muscle troponin I isoform is expressed in

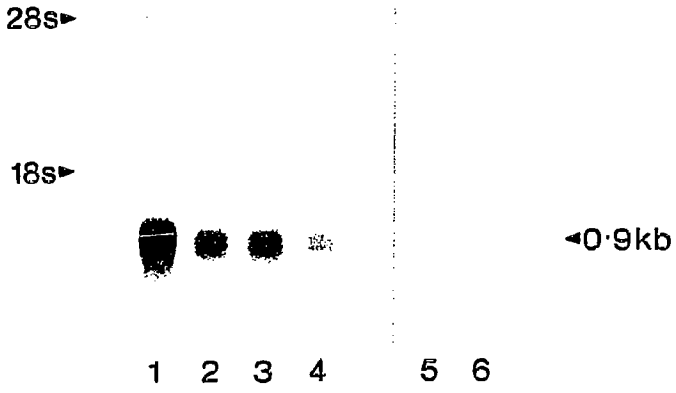

Fig. 2. Detection of slow TnI mRNA in fetal heart. RNA from fetal hearts of ages 8 weeks (lane 1), 12 weeks (lane 2), 14 weeks (lane 3) and 28 weeks (lane 4) together with fetal skeletal muscle (lane 5) and adult atrial muscle (lane 6) was analysed by Northern blotting. Following hybridization with a PCR-derived probe specific for TnIs mRNA (see text), a single band is seen in both fetal heart and fetal skeletal muscle samples confirming the presence of TnIs mRNA in the fetal (but not adult) heart.

human fetal heart, and that it constitutes the predominant troponin I isoform in early cardiac development.

3.2. *Detection of mRNA for slow troponin I in fetal heart*

If TnIs is expressed in the fetal heart then mRNA encoding this isoform should be present. In order to detect slow TnIs mRNA, we made a PCR-derived cDNA fragment corresponding to the 3′ non-coding region of the TnIs mRNA using published sequence data [11]. This region of the TnIs sequence has previously been demonstrated to be isoform-specific [11] but in order to verify the specificity of our PCR-derived cDNA fragment, we carried out Southern blot hybridization to digests of human genomic DNA. This resulted in the same pattern of single-copy bands (data not shown) as previously described for the TnIs gene [11].

Fig. 2 shows a Northern blot of total RNA extracts from several fetal heart samples (8-, 12-, 14- and 28-week), fetal skeletal muscle and adult atrial muscle, following hybridization with the TnIs cDNA probe. A single mRNA band is seen in all the fetal heart tracks as well as with fetal skeletal muscle, thereby confirming the presence of TnIs mRNA in fetal heart. No hybridization is seen with adult atrial RNA. The level of signal shows an apparent decrease with developmental age in the fetal heart samples.

3.3. *Timing of transitions in troponin I expression*

To further investigate the transition in accumulation from TnIs to TnIc we examined a series of fetal and postnatal heart samples. Fig. 3 shows samples analysed on Western blots with troponin isoforms detected with antibody 42/25. As expected from our initial observations, all early fetal samples contain a single predominant protein band corresponding to TnIs. Samples taken from 33-week fetal and 15-day neonatal heart contain TnIs with increasing amounts of TnIc. However, the 9-month postnatal heart sample contained only TnIc. This demonstrates a transition in troponin expression which occurs between 20 weeks fetal development and 9 months postnatal life and which involves increased accumulation of TnIc and the loss of TnIs over this period.

4. DISCUSSION

We have demonstrated that there is a transition in expression of troponin I isoforms during fetal development of the human heart from slow TnI (TnIs) which is the major isoform in early fetal heart, to cardiac troponin I (TnIc), the adult isoform. This transition occurs over a period from 20 weeks gestation and 9 months postnatal development. Expression of slow skeletal muscle troponin I in fetal heart has been previously described for rat and chick and would appear to be a common feature of cardiac development in vertebrates.

The troponin complex is involved in the regulation of striated muscle contraction and troponin I probably acts as a molecular switch which regulates the interaction of actin and myosin in response to calcium (e.g. [24]). As TnIs and TnIc are structurally different, transition in expression from TnIs to TnIc during fetal development is of importance when considering contractility in the developing heart. TnIc has an extended N-terminal sequence which contains two serine residues which are phosphorylated in response to adrenergic stimulation. Phosphorylation at these sites is probably effected by cAMP-dependent protein kinase and results in altered contractile properties of the myocardium (reviewed in [25]) including increased rate of relaxation [26]. Our data suggest that early fetal heart contains predominantly TnIs. As this isoform lacks the N-terminal serine residues involved in this phosphorylation it would be expected to confer reduced responsiveness to adrenergic stimulation and the developmental transition to expression of the adult TnIc isoform should result in a developmental increase in responsiveness. The molecular mechanisms which effect changes in tro-

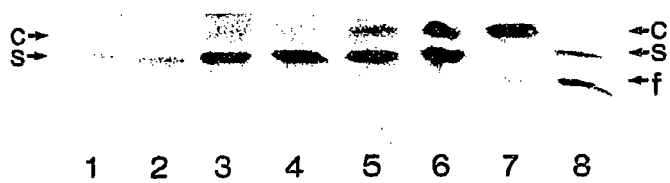

Fig. 3. Transition in troponin I expression during fetal cardiac development. Immunoblot of protein extracts from fetal heart at 8 weeks (lane 1), 11 weeks (lane 2), 14 weeks (lane 3), 20 weeks (lane 4) and 33 weeks (lane 5) gestation, 15-day (lane 6) and 9-month (lane 7) postnatal heart and fetal skeletal muscle (lane 8), following detection with antibody 42/25, C, S and F indicate the positions of TnIc, TnIs and TnIf, respectively.

ponin I expression in the heart are unknown although developmental changes in hormone levels, in particular thyroid hormone, may play a direct role [27]. Further experiments aimed at elucidating the exact timing and level of control are needed to address this issue.

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REFERENCES

[1] Leszyk, J., Dumaswala, R., Potter, J.D. and Collins, J.H. (1988) Biochemistry 27, 2821–2827.
[2] Wilkinson, J.M. and Grand, R.J.A. (1978) Nature 271, 31–35.
[3] Cummins, P. and Perry, S.V. (1978) Biochem. J. 171, 251–259.
[4] Dhoot, G.K. and Perry, S.V. (1979) Nature 278, 714–718.
[5] Saggin, L., Gorza, L., Ausoni, S. and Schiaffino, S. (1989) J. Biol. Chem. 264, 16299–16302.
[6] Sabry, M.A. and Dhoot, G.K. (1989) J. Mus. Res. Cell Motil. 10, 85–91.
[7] Dhoot, G.K., Gell, P.G.H. and Paerry, S.V. (1978) Exp. Cell Res. 117, 357–370.
[8] Vallins, W.J., Brand, N.J., Dabhade, N., Butler-Browne, G.S., Yacoub, M.H. and Barton, P.J.R. (1990) FEBS Lett. 270, 57–61.
[9] Nikovits, W., Kuncio, G. and Ordahl, C.P. (1986) Nucleic Acids Res. 14, 3377–3390.
[10] Koppe, R.I., Hallauer, P.L., Karpati, G. and Hastings, K.E.M. (1989) J. Biol. Chem. 264, 14327–14333.
[11] Wade, R., Eddy, R., Shows, T.B. and Kedes, L. (1990) Genomics 7, 346–357.
[12] Baldwin, A.S., Kittler, E.L.W. and Emerson, C.P. (1985) Proc. Natl. Acad. Sci. USA 82, 8080–8084.
[13] Murphy, A.M., Jones, L., Sims, H.F. and Straus, A.W. (1991) Biochemistry 30, 707–712.
[14] Solaro, R.J., Moir, A.J.G. and Perry, S.V. (1976) Nature 262, 615–617.
[15] Swiderek, K., Jacquet, K., Meyer, H.E. and Heilmyer, M.G. (1988) Eur. J. Biochem. 176, 335–342.
[16] Mittmann, K., Jaquet, K. and Heilmeyer, L.M.G. (1990) FEBS Lett. 273, 41–45.
[17] Toyota, N. and Shimada, Y. (1981) J. Cell Biol. 91, 497–504.
[18] Humphreys, J.E. and Cummins, P.J. (1984) J. Mol. Cell. Cardiol. 16, 643–657.
[19] Dhoot, G.K., Drainsfield, I., Grand, R.J.A. and Perry, S.V. (1986) J. Mus. Res. Cell Motil. 7, 351–360.
[20] Bradford, M.B. (1976) Anal. Biochem. 72, 248–254.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[23] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning – a Laboratory Manual, 2nd edn., Cold Spring Harbour Lab. Press, New York.
[24] Tao, T., Gong, B.J. and Leavis, P.C. (1990) Science 247, 1339–1341.
[25] Winegrad, S. (1984) Circ. Res. 55, 565–574.
[26] Robertson, S.P., Johnson, D.J., Holyroyde, M.J., Kranias, E.G., Potter, J.D. and Solaro, R.J. (1982) J. Biol. Chem. 257, 260–263.
[27] Diekman, L.J. and Solaro, R.J. (1990) Circ. Res. 1990, 344–351.