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Neural cell adhesion molecule (N-CAM) in fetal and mature human heart

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Using Northern blot analysis and immunoblotting techniques we report for the first time, that the neural cell adhesion molecule, N-CAM, is expressed in human heart. We found several different N-CAM transcripts in human fetal (13–17 weeks gestation) and mature heart (left ventricle from a 5-year-old child). Northern blotting showed that a 5.2 kb transcript was the most abundant and progressively increased with age, both in fetal and mature heart. These transcripts may correspond with the different protein isoforms shown by immunoblotting. We also confirmed the presence of N-CAM in fetal and mature myocytes by immunohistochemical techniques, using a monoclonal antibody to human N-CAM. Results demonstrated that N-CAM is mainly confined to the myocyte cell surface.

N-CAM; Heart; Fetal human; Northern blotting

1. INTRODUCTION

Neural cell adhesion molecule (N-CAM) is one of a family of cell surface sialoglycoproteins which facilitates cell-cell adhesion [1,2]. It is predominantly expressed in neurons and striated muscle, but has also been shown to be present in cardiac myocytes both in the mouse [3] and the rat [4]. A number of distinct isoforms have been described and their pattern of expression is known to be tissue-dependent [5]. This is accomplished from a single copy gene by alternative RNA processing at the levels of splicing and polyadenylation. Three isoforms are present each in brain [6] (180 kDa, 140 kDa and 120 kDa) and skeletal muscle [9] (155 kDa, 145 kDa, 125 kDa). The presence of two isoforms (150 kDa and 125 kDa) has been demonstrated in embryonic mouse heart; the larger component is lost soon after birth [3]. Two isoforms (145 kDa and 105 kDa) are expressed in embryonic rat heart [4] with two new forms, 155 kDa (transiently) and 125 kDa (permanently), appearing after birth. In developing rodent brain and skeletal muscle, the appearance of the 120 kDa and 125 kDa isoforms has been linked to mRNA species of 2.9 and 5.2 kb [8,9]. N-CAM levels are known to respond to physiological stimuli; although high levels of N-CAM are expressed in embryonic muscle, they are lost from extrajunctional sarcolemma in adult skeletal muscle. However, after denervation, N-CAM protein and mRNA expression is restimulated leading to the generalized distribution of N-CAM to muscle sarcolemma [8]. No previous study has demonstrated the presence of N-CAM isoforms and transcripts in fetal or mature human myocardium. The present study was designed to describe, in developing human heart, the temporal pattern of the relative levels of N-CAM expression in terms of its protein isoforms and mRNA transcripts and to demonstrate, immunohistochemically, the spatial distribution of myocyte N-CAM localization.

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2. MATERIALS AND METHODS

2.1. *Northern blot analysis of N-CAM mRNA*

RNA was extracted from heart by homogenization in 6 M guanidinium isothiocyanate solution followed by CsCl ultracentrifugation [10]. Samples of total RNA were extracted from human fetal hearts of various gestational ages and from the left and right ventricles of a 5-year-old patient with cystic fibrosis at the time of heart-lung transplantation. RNA was first denatured with formamide and formaldehyde, separated by electrophoresis on 1.5% agarose gels in formaldehyde and 1 × Mops buffer. Samples of RNA were transferred to nylon filters (Genescreen, DuPont) in 25 mM NaH_2PO_4/Na_2HPO_4 (pH 6.5). Filters were then washed with the same buffer for 15 min and prehybridized for 16 h at 42°C in 50% formamide, 0.04% polyvinylpyrollidone, 0.04% BSA, 0.04% Ficoll, 5 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 1% SDS, 200 μg/ml sheared, denatured salmon sperm DNA (Sigma Chemical Co.) and 2 μg/ml polyA and 1 μg/ml polyG (Sigma Chemical Co.). Hybridization was carried out in the same solution for 20 h with an electrophoretically purified insert from the human muscle N-CAM cDNA clone λ9.5 [7] labelled with [^{32}P]deoxycytidine triphosphate (Amersham). Filters were washed with 2 × SSC, 0.1% SDS for 1 h at 42°C followed by washes at 2 × SSC, 1% SDS twice for 30 min at 65°C and then by 0.1 × SSC for 15 min at 20°C. Filters were then exposed to Kodak X-ray film (Xomat 5AR) at −70°C in the presence of an intensifying screen.

2.2. *Preparation of N-CAM protein and immunoblotting*

The procedure of Moore et al. [9] was followed with minor modifications. Briefly, the heart tissues were homogenized in 1% NP-4, solubilized in 20 mM Tris-HCl, 0.85% NaCl, pH 7.4, con-

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taining 2 U/ml Trasylol (Sigma) and 1 mM PMSF (Sigma) as protease inhibitors. The homogenate was then centrifuged at 100000 × *g* for 1 h at 4°C and the soluble supernatant was collected. The soluble fraction was then treated, for 1 h at 37°C, with neuraminidase (type X, Sigma) at a final concentration of 0.5 U/ml in 2.5 mM sodium acetate buffer, pH 5.0, to generate the desialo forms of N-CAM. The reaction was terminated by the addition of gel loading sample buffer [11] containing SDS and 2-mercaptoethanol. Polyacrylamide gel separation was performed according to the procedure described by Laemmli [11], using 8.5% polyacrylamide gel. The resolved proteins were then electroblotted onto nitrocellulose (NC) membrane (Schleisher and Shuell, Anderman and Co.) according to the protocol of Towbin et al. [12], and treated with a monoclonal antibody to human N-CAM.

2.3. *Tissue processing and immunofluorescence staining*

Cryostat sections (10–15 μm) were air-dried for 1 h, then fixed for 10 min in methanol/acetone (1:1). Sections were briefly washed in PBS and then immersed in Pontamine sky blue [13] for 30 min at room temperature. They were then washed with several changes of PBS for 45 min. Anti-human N-CAM mouse monoclonal antibody was added at a dilution of 1/100 for 30 min at room temperature. The slides were then washed with PBS (3 changes) for 30 min. A second antibody, sheep anti-mouse whole Ig conjugated to FITC (Sigma), was added and the slides were incubated for 1 h at room temperature. They were extensively washed with several changes of PBS. The slides were mounted and visualized. Control sections were processed in the same way except using normal mouse serum as the first antibody.

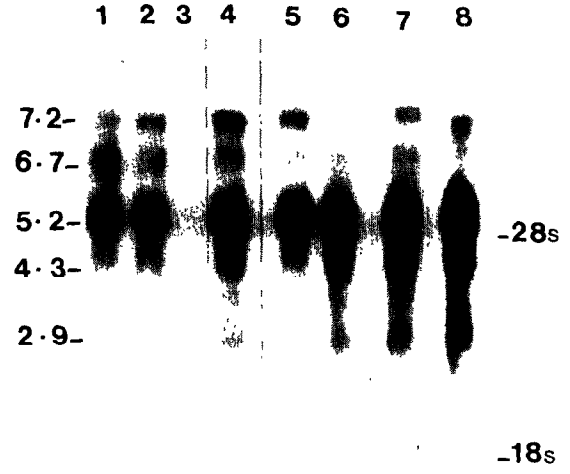

Fig. 1. Northern blot analysis of human cardiac N-CAM, with the whole λ9.5 cDNA probe, using 10 μg of total RNA from human fetal and mature human heart. (Lane 1) 13.4 weeks gestation; (lane 2) 14.3 weeks gestation; (lane 3) a lymphoblastoid cell line; (lane 4) 15.5 weeks gestation; (lane 5) 16.3 weeks gestation; (lane 6) 16.6 weeks gestation; (lane 7) 17 weeks gestation; and (lane 8) left ventricle from a 5-year-old cystic fibrosis transplant. The positions of the 28 S and 18 S rRNA standards are shown on the right, and the sizes of N-CAM transcripts are indicated on the left in kb. Gestation ages were calculated by the Royal Marsden Tissue Bank.

3. RESULTS AND DISCUSSION

Fig. 1 shows the Northern blot analysis of RNA from human heart of various fetal ages (13–17 weeks) as well as that derived from mature left ventricular myocardium (mature right ventricle gave a similar pattern; results not shown) and probed with the human muscle N-CAM cDNA probe, λ9.5. We found at least 5 transcripts; these were of 7.2, 6.7, 5.2, 4.3 and 2.9 kb, respectively. These transcripts represent a full complement corresponding to those previously described in mouse brain [14], although only the 7.2, 6.7 and 4.3 kb transcripts appear during embryonic life [5] with the 5.2 and 2.9 transcripts appearing only postnatally. N-CAM transcript size has not previously been reported in heart for any mammalian species. However, Prediger et al. [15] have described mRNA transcripts of 6.4, 4.3 and 3 kb in chicken cardiac tissue. We not only report that all 5 transcripts are expressed in fetal and mature human heart, but also show that the level of expression, particularly of the 5.2 kb transcript, progressively increased with gestational age and had increased even more in mature myocardium. This pattern of development may be related to the hypoplastic requirement and progressive differentiation of embryonic myocytes; a process which is complete and ceases soon after term.

Fig. 2 (Western blotting) shows the reaction pattern of the desialylated N-CAM isoforms using the immunoblotting technique. All fetal samples showed similar reactive protein bands at apparent molecular masses of 140, 135, and 120 kDa (Fig. 2, lanes 2–5). In contrast, adult heart left ventricle (LV), revealed only two immunoreactive bands with the anti-human N-CAM antibody. These two polypeptides are of apparent molecular masses of 125 and 105 kDa (Fig. 2, lane 6) with the larger polypeptide giving the strongest signal. A mouse fibroblast cell line (3T3) transfected with human N-CAM gave only the 125 kDa band. Thus, we found that human heart showed a different pattern of N-CAM expression to that in rat heart [4]

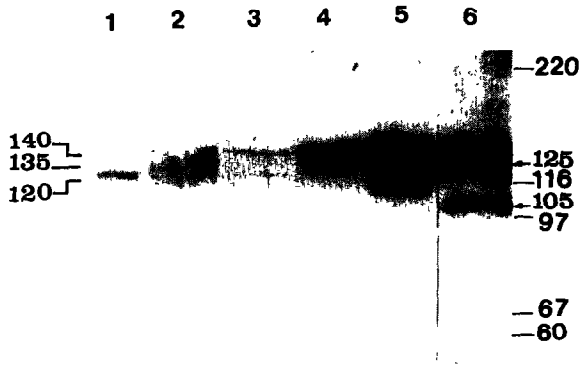

Fig. 2. Western blot analysis of neuraminidase-treated desialo N-CAM isoforms in fetal and mature human heart tissue. (Lane 1). 3T3 mouse fibroblast cell line transfected with human N-CAM; (lane 2) 11.4 weeks gestation; (lane 3) 14.0 weeks gestation; (lane 4) 14.8 weeks gestation; (lane 5) 16.6 weeks gestation; (lane 6) left ventricle from a 5-year-old cystic fibrosis transplant. Molecular mass markers are shown on the right; sizes of N-CAM polypeptides from fetal, on the left, and from mature hearts (arrows) on the right (in kDa).

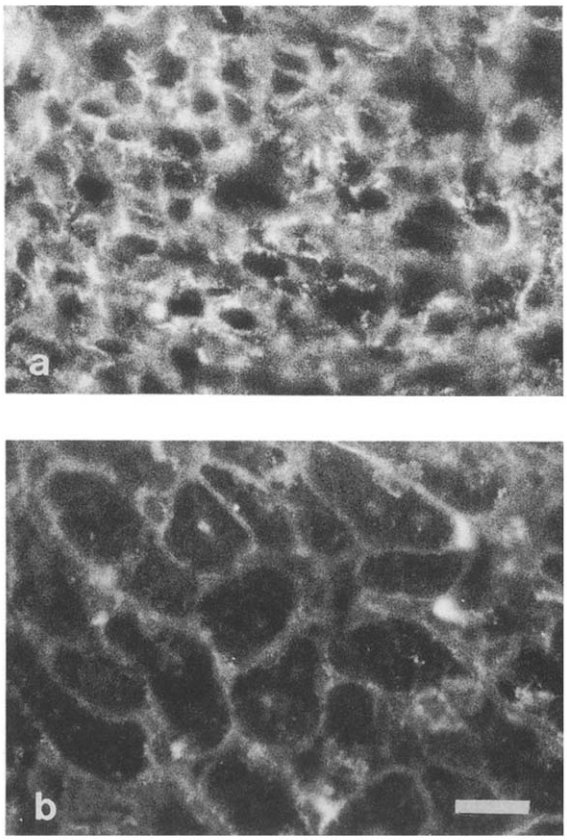

Fig. 3. N-CAM immunofluorescence staining in fetal and mature human myocardium. (a) Fetal myocytes; 14.0 weeks gestation. (b) Mature myocytes; left ventricle from a 5-year-old transplanted for cystic fibrosis. Bar = 10 μm.

where, after the appearance during embryonic life of N-CAM isoforms corresponding to 105 and 145 kDa, they are replaced postnatally by isoforms of 125 and 155 kDa. A similar pattern has been found during development in mouse skeletal muscle [9]. As in these studies, we also noted the postnatal appearance of a 125 kDa isoform, but we found all fetal isoforms to be of different sizes when comparing rodent and human. In the mouse, the expression of the N-CAM isoforms is believed to be controlled on the gene level by alternative splicing in combination with the use of different polyadenylation sites [16]. In the present study, fetal heart N-CAM shows 3 isoforms, with two different isoforms appearing in mature myocardium. This does not reflect a post-translational glycosylation difference since the samples were desialylated prior to their reaction with the anti-human N-CAM monoclonal antibody.

Cardiac cells exhibited intense immunofluorescent staining along the sarcolemma, using anti-human N-CAM monoclonal antibody, on frozen and fixed sections at 14 weeks gestation (and beyond) (Fig. 3a). A similar reaction was also noted in sections from mature heart (Fig. 3b). The validity of these immunofluorescence studies was confirmed using immunoperoxidase staining (results not shown). A similar sarcolemmal pattern of N-CAM localization has recently been reported [4] in developing rat heart. In both rat and human, there is clearly a different level of appropriate N-CAM expression during the various stages of development in fetal and mature myocardium. In part, this may reflect the postnatal change from dividing to non-dividing myocytes and the development of a fully mature syncytium. The difference in N-CAM expression may also be related to the postnatal development of the adrenergic supply to the heart. What is clear is that N-CAM is subject to temporal regulation in the developing heart and that the precise pattern of expression differs markedly between species.

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