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Development of the peptidergic innervation of human heart

L. GORDON[1], J. M. POLAK[1], G. J. MOSCOSO[2], A. SMITH[3], D. M. KUHN[4] AND J. WHARTON[1]

[1] *Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital,* [2] *Department of Morbid Anatomy, King's College Hospital, London, and* [3] *Institute of Child Health, University of Liverpool, UK, and* [4] *Laboratory of Neurochemistry, Lafayette Clinic, Detroit, USA*

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ABSTRACT

The aim of the present investigation was to study the developing peptidergic innervation of the human fetal heart of 7–24 wk gestational age. An immunohistochemical approach was adopted and the total innervation visualised with antisera to general neuronal and Schwann cell markers, while the onset and development of specific neuropeptide-containing subpopulations were investigated using antisera to neuropeptide Y (NPY), somatostatin, vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP) and substance P (SP). Cardiac ganglia and nerves were demonstrated from 7 wk of gestation whereas peptide-immunoreactive nerves were not observed until the 10th week of gestation. NPY-immunoreactive nerve fibres constituted the major subpopulation of peptide-containing nerves identified in the fetal heart, exhibiting a descending atrial to ventricular density gradient, and were first identified during the 10th wk of gestation. Somatostatin- and VIP-immunoreactive nerves appeared at 10–12 wk of gestation and were mainly distributed in the atria. Somatostatin immunoreactivity was localised to cell bodies in cardiac ganglia, as well as to nerve fibres, indicating an intrinsic origin for this nerve subpopulation. Conversely, the other peptide-containing nerves appear to be of extrinsic origin, including those immunoreactive for VIP. Intracardiac neurons exhibit a transient expression of tyrosine hydroxylase immunoreactivity. Putative sympathetic nerve fibres, displaying tyrosine hydroxylase and NPY immunoreactivity, were demonstrated before the adrenergic innervation has previously been shown to be present by formaldehyde-induced fluorescence staining of catecholamines. The onset of the CGRP- and SP-immunoreactive innervation, at 18–24 wk of gestation, followed the appearance of other peptide-containing nerves, suggesting that the sensory, afferent innervation occurs later than the autonomic. The differential appearance and distribution of peptide-containing nerve subpopulations indicate that there is a chronological order to the development of the autonomic and sensory components of human cardiac innervation.

INTRODUCTION

Histological and ultrastructural studies have demonstrated the presence of developing neurons and nerves in the human heart at 5–6 wk of gestation (Navaratnam, 1965*a*; Smith, 1970, 1971; Kanerva et al. 1974; Gardner & O'Rahilly, 1976; Shvalev & Sosunov, 1989). Other studies, using formaldehyde-induced fluorescence and acetylcholinesterase staining techniques, have revealed numerous putative cholinergic neurons but a paucity of adrenergic nerve fibres in the human fetal heart of 8–18 wk of gestation (Navaratnam, 1965*b*; Taylor & Smith, 1971; Dail & Palmer, 1973; Partanen & Korkala, 1974), suggesting that the various components of cardiac innervation may develop at different rates.

In addition to the presence of the classical neurotransmitters noradrenaline and acetylcholine, subpopulations of autonomic and sensory cardiac nerves also contain immunohistochemically-defined neuropeptides, such as neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), somatostatin, calcitonin gene-related peptide (CGRP) and substance P (SP), which elicit potent effects on cardiac function and/or coronary blood flow (Wharton & Gulbenkian, 1987). While a number of studies have demonstrated the

Correspondence to Dr John Wharton, Department of Histochemistry, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK.

presence and differential distribution patterns of peptide-containing nerves in the adult human heart (Pujeranta et al. 1986; Rechardt et al. 1986; Wharton et al. 1988, 1990), information regarding the onset and development of the cardiac peptidergic innervation is lacking.

In the present study the development of the peptidergic innervation was investigated, by the use of immunofluorescence staining, in a series of human fetal hearts of 7–24 wk gestational age. Peptide-containing nerves were examined in the context of the developing general innervation, as defined by neuronal and Schwann cell markers, and with reference to neurons containing the catecholamine-synthesising enzyme tyrosine hydroxylase.

MATERIALS AND METHODS

The development of the cardiac innervation was investigated in a series of human fetal hearts (n = 31), ranging in gestational age from 7–8 (n = 4), 9–12 (n = 8), 13–16 (n = 7), 17–20 (n = 6) to 21–24 wk (n = 6). The organs were obtained following miscarriage or legal abortion by uterine evacuation for reasons other than suspected cardiac abnormality.

Gestational age was determined by measurements of fetal crown–rump and foot length, together with details of the maternal history. The hearts were processed as intact organs or dissected and the tissues immersion-fixed in Zamboni's solution for approximately 6 h followed by several washes in the cryoprotectant phosphate buffered saline (PBS)-sucrose (15% w/v). Cryostat blocks were prepared by orienting the tissue on cork mats, covering in Tissue-Tek (Miles Inc., USA) and freezing in melting dichlorodifluoromethane suspended in liquid nitrogen. Each piece of tissue was then stored in liquid nitrogen prior to cutting sections and immunostaining by the indirect immunofluorescence method (Wharton et al. 1988). Briefly, 15 μm thick frozen sections were cut at −25 °C, mounted on poly-L-lysine-coated slides and air dried for 1 h at room temperature. After permeabilisation of the tissue with PBS containing 0.2% v/v Triton X-100 (BDH, Poole, UK) for 30 min, the sections were counterstained with pontamine sky blue (BDH) to reduce tissue autofluorescence. Sections were then incubated with primary antisera (Table 1) at appropriate dilution for 16–24 h at 4 °C, followed by the application of fluorescein isothiocyanate-conjugated IgG for 1 h at room temperature. The preparations were mounted in PBS: glycerol (2:1) and examined with an Olympus AH-2 microscope equipped for epi-illumination. The developing cardiac innervation was visualised by the localisation of immunoreactivity for the general neural markers protein gene product 9.5 (PGP 9.5), neurofilaments, synaptophysin and neural cell adhesion molecule (N-CAM) and the Schwann cell marker S-100. The development of peptide-containing nerve subpopulations was investigated by the localisation of immunoreactivity for NPY, somatostatin, VIP, CGRP and SP. The distribution of peptide-containing nerves was also compared with that of nerves displaying tyrosine hydroxylase immunoreactivity. All the antisera have been well characterised in previous studies (Wharton et al. 1988, 1990; Gordon et al. 1990) and their origins are detailed in Table 1. Controls included the omission of the primary antiserum or its substitution with preimmune serum and/or preabsorption with appropriate antigen in the range 10^{-5}–10^{-8} M.

Table 1. *Primary antibodies*

Antigen	Code	Dilution	Source
PGP 9.5	1648 (RA95103)	1:2000	Ultraclone, UK
S-100	1508 (Z311)	1:800	DAKO, UK
Synaptophysin	1623 (G95)	1:800	R. Jahn, Germany
Neurofilaments	1472	1:800	D. Dahl, USA
N-CAM	1701	1:500	G. M. Edelman, USA
TH	1498 (KD1-1)	1:400	D. M. Kuhn, USA
NPY	1411 (JN7)	1:600	Hammersmith Hospital
VIP	652 (V128)	1:2000	Hammersmith Hospital
Somatostatin	1082 (70H2T)	1:400	Immuno Nuclear, USA
CGRP	1208 (CGRP-7)	1:200	Hammersmith Hospital
Substance P	910 (SP35)	1:500	Hammersmith Hospital

PGP 9.5, protein gene product 9.5; N-CAM, neural cell adhesion molecule; TH, tyrosine hydroxylase; NPY, neuropeptide Y; VIP, vasoactive intestinal polypeptide; CGRP, calcitonin gene-related polypeptide.

RESULTS

General innervation

The progressive development of the general cardiac innervation was observed from 7 wk of gestation up to and including the 24th wk, as defined by immunoreactivity for neural and Schwann cell markers. By 7–10 wk of gestation, nerves and ganglia displaying PGP 9.5, neurofilament and S-100 immunoreactivity were localised to the atrial epicardium and adventitia of the aorta and pulmonary trunk, and immuno-

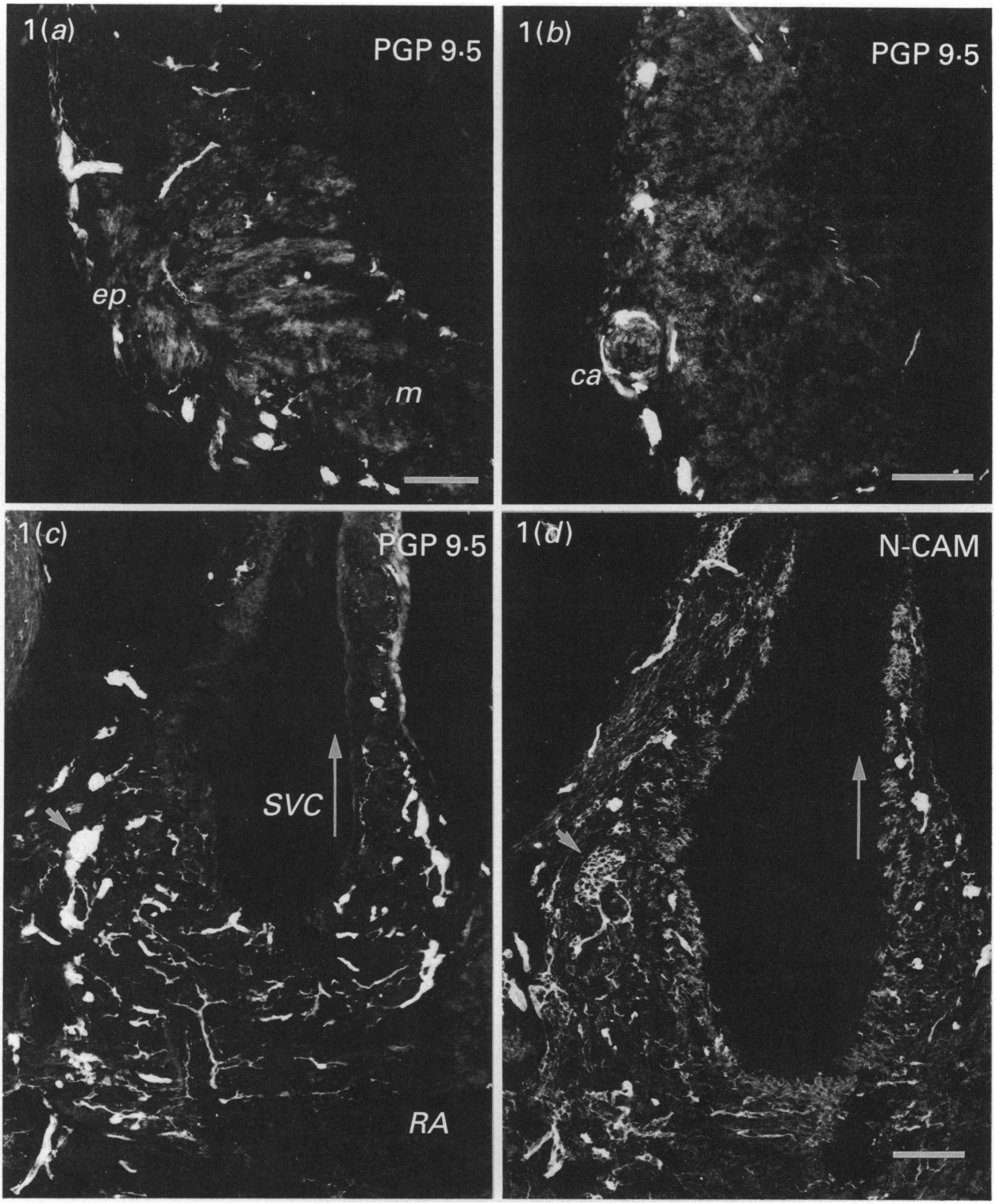

Fig. 1. Protein gene related peptide 9.5 (PGP 9.5) was localised to coarse nerve fascicles in (*a*) the atrial epicardium (*ep*) and myocardium (*m*) and (*b*) around a coronary artery (*ca*) in the left ventricular epicardium at 9 wk of gestation. An extensive network of PGP 9.5-immunoreactive nerves and ganglia (*c*) were observed in the sinus node at 8 wk of gestation (*SVC*, superior vena cava; *RA*, right atrium) and a similar pattern of neural immunostaining was observed in a serial section (*d*) with antiserum to neural cell adhesion molecule (N-CAM). The same cardiac ganglion is indicated by an arrow in panels (*c*) and (*d*). Bar, 50 μm.

reactive nerves had penetrated the sinus node (Fig. 1). Nerves and ganglia which were PGP 9.5-immunoreactive also exhibited immunoreactivity for the cell adhesion molecule N-CAM. The ventricles were innervated later than the atria and PGP 9.5-immunoreactive nerves were localised around the

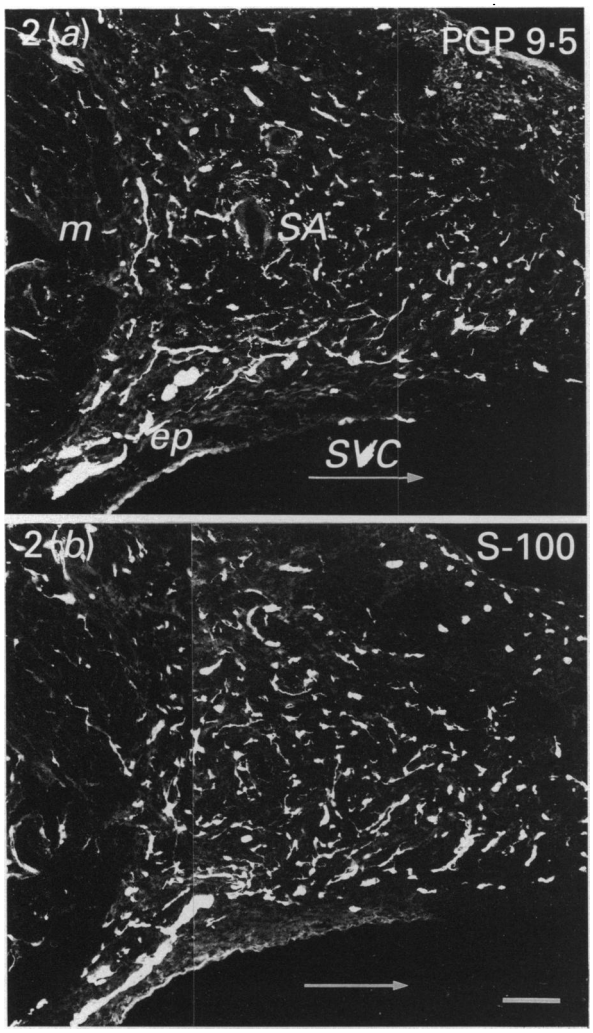

Fig. 2. PGP 9.5-immunoreactive nerves (*a*) and S-100-immunoreactive Schwann cells (*b*), were localised in serial sections of sinus node at 18 wk of gestation. *SA*, sinus node; *ep*, epicardium; *SVC*, superior vena cava; *m*, atrial myocardium. Bar, 50 μm.

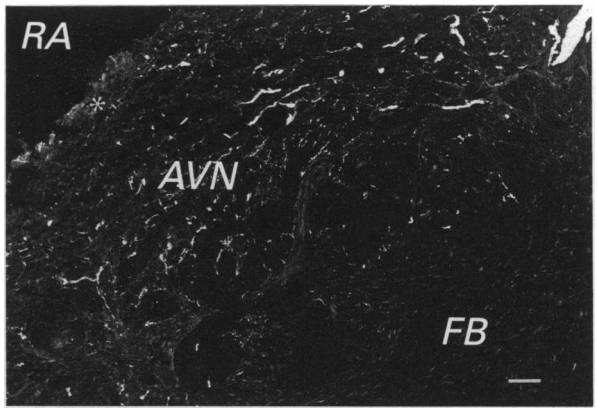

Fig. 3. PGP 9.5-immunoreactive nerves localised in the atrioventricular node at 15 wk of gestation. *AVN*, atrioventricular node; *FB*, fibrous body; *RA*, right atrium; asterisk, atrial myocardium overlying the node. Bar, 50 μm.

Fig. 4. Synaptophysin immunoreactivity is localised in varicose nerve fibres (fine arrows), associated with nonimmunoreactive ganglionic cell bodies, and in paraganglionic cells bodies (bold arrows) lying between the aorta and pulmonary trunk, at 18 wk of gestation. Nerve trunks (*n*) are also nonimmunoreactive. Bar, 50 μm.

epicardial coronary vessels in the ventricles at 9 wk of gestation (Fig. 1). At 12–14 wk of gestation nerve fibres and fascicles were increasingly distributed throughout the atrial myocardium and endocardium and around intramyocardial blood vessels. During this interval the perivascular plexus of ventricular coronary arteries extended with the vessels from the epicardium into the myocardium. The innervation density continued to increase up to 24 wk at which time the distribution pattern resembled that observed in the adult. Throughout the series of hearts examined the innervation of the sinus and atrioventricular nodes was found to be greater than in the adjacent myocardium whereas the innervation density of the ventricular conduction system appeared to be no greater than that of the lateral ventricle walls (Fig 2, 3).

The development of the terminal innervation in the heart was visualised by immunostaining of varicose nerve fibres for synaptophysin and a similar pattern to that described above was observed during the 7–24 wk period. Immunoreactive varicose fibres were prominent at 18 wk of gestation, intimately associated with intrinsic cardiac ganglion cells which were non-immunoreactive (Fig. 4). Adjacent nerve trunks were also nonimmunoreactive, whereas clusters of paraganglion cells displayed prominent cytoplasmic immunoreactivity for synaptophysin.

Peptide-containing nerves

Neuropeptide immunostaining was not detected in either nerve fibres or neurons until the 10th week of gestation. NPY immunoreactivity was first demon-

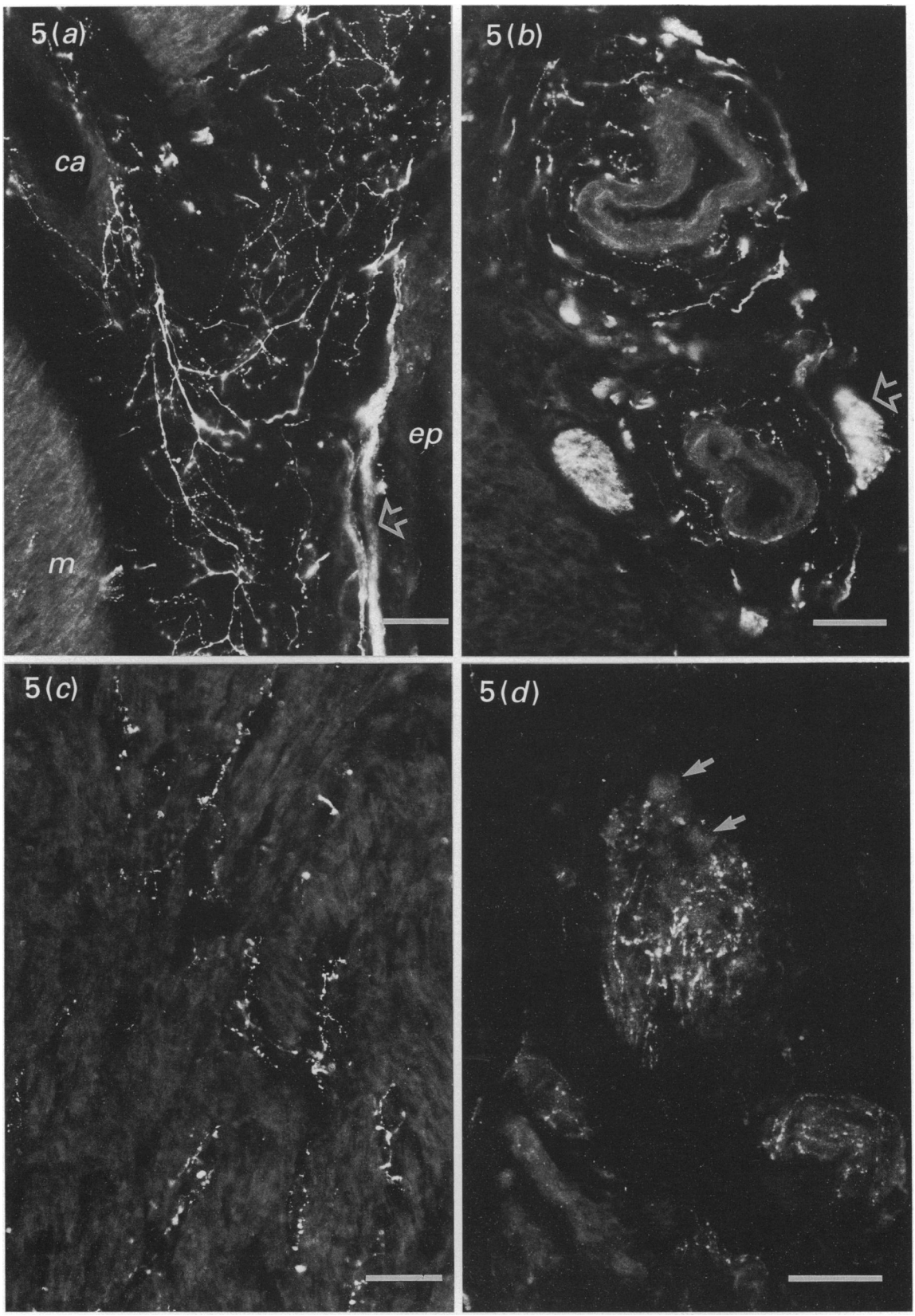

Fig. 5. Neuropeptide Y-immunoreactive nerve fibres (*a*) in a perivascular plexus in the left ventricular epicardium (*ep*) at 19 wk (*ca*, coronary artery; *m*, myocardium); (*b*) associated with the adventitia of the left anterior descending coronary artery; (*c*) scattered throughout the myocardium of the interventricular septum at 23 wk; and (*d*) around nonimmunoreactive ganglion cell bodies (arrows) at 18 wk of gestation. Open arrows, intensely immunostained nerve bundles. Bar, 50 μm.

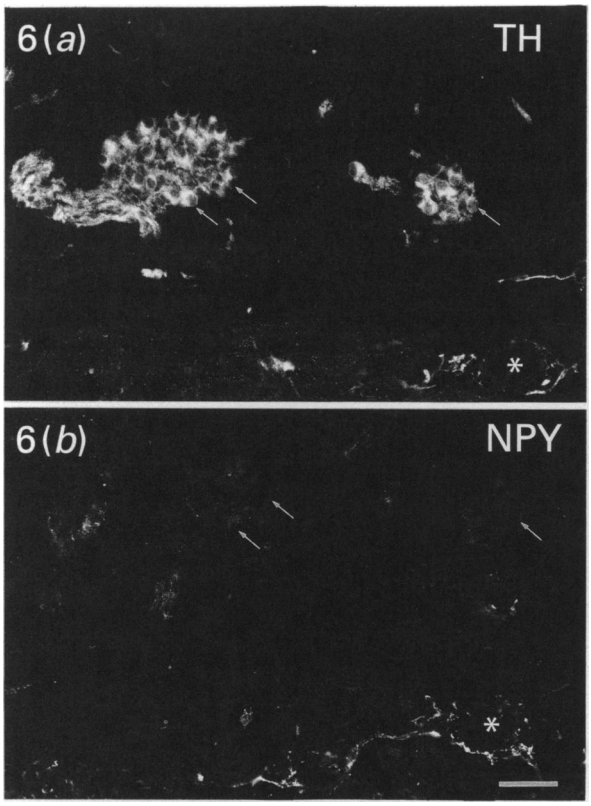

Fig. 6. Tyrosine hydroxylase (TH) immunoreactivity is localised to intrinsic neurons in the atrial epicardium at 18 wk of gestation (*a*) while, in a serial section (*b*) immunostained for neuropeptide Y (NPY) the ganglion cell bodies (arrows) are nonimmunoreactive. TH and NPY-immunoreactive nerve fibres are present in the myocardium and associated with intramyocardial coronary arteries (asterisks). Bar, 50 μm.

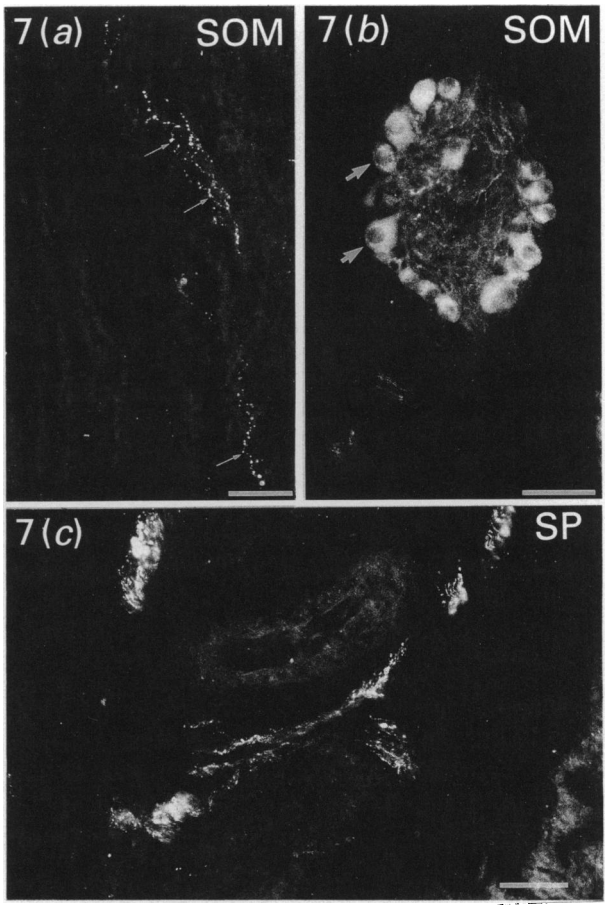

Fig. 7. Somatostatin (SOM)-immunoreactive varicose nerve fibres (fine arrows) are present in the right atrial myocardium (*a*) and in the cell bodies (bold arrows) in a cardiac ganglion in the atrial epicardium (*b*) of a heart of 23 wk of gestation. Substance P (SP) immunoreactivity is localised to nerve fascicles in the atrioventricular groove (*c*) at 23 wk of gestation. Bar, 50 μm.

strated in nerve fibres localised to the atrial epicardium at about 10 wk of gestation and was immediately followed by the appearance of somatostatin and VIP-immunoreactive nerve fibres at 10–12 wk of gestation. In contrast, nerve fibres displaying CGRP and SP immunofluorescence staining were not detected until 18–24 wk of gestation.

NPY-immunoreactive nerve fibres represented the predominant peptide-containing nerve population in all the fetal hearts examined. By 14–18 wk of gestation these fibres were distributed throughout the atrial myocardium and formed distinct epicardial and perivascular plexuses in the ventricles (Fig. 5). The density of innervation increased up to 24 wk of gestation and at this time immunoreactive nerve fascicles and fibres were localised throughout the endocardial and epicardial layers and formed an extensive innervation at the adventitial-medial border of both large and small intramyocardial blood vessels, extending with the coronary vasculature into the myocardium (Fig. 5). Whilst NPY immunoreactivity was not observed in ganglionic cell bodies, numerous immunoreactive nerve fibres were intimately associated with them (Fig. 5). By contrast, tyrosine hydroxylase immunoreactivity was detected in the cell bodies of ganglia and paraganglia, as well as nerve fascicles (Fig. 6). At 7–10 wk of gestation immunoreactivity was confined to ganglia, paraganglia and nerve trunks associated with the aorta and pulmonary trunk and to a few fascicles in the right atrial epicardium. The density of tyrosine hydroxylase-immunoreactive nerves gradually increased with gestational age and by the 14th week immunoreactive varicose fibres were observed throughout the myocardium, but were more extensive in the atria compared with the ventricles. In the latter stages of fetal development ganglion cell immunostaining was equivocal.

Somatostatin-immunoreactive nerve fibres were first detected between 10 and 12 wk of gestation and were predominantly localised to the atria. At this time they had also started to invade both the sinus and

atrioventricular nodes. Scattered somatostatin-immunoreactive fibres were identified in the atrial myocardium (Fig. 7), endocardium and epicardium whereas ventricular immunoreactivity was particularly sparse. Somatostatin immunoreactivity was also localised to a large proportion of the cell bodies in cardiac ganglia present in the subepicardium and adventitia of the great vessels in second trimester fetuses (Fig. 7). Similarly, VIP-immunoreactive nerve fibres were first demonstrated between 10 and 12 wk, although they remained relatively sparse up to and including 24 wk of gestation. Immunofluorescent nerves were localised throughout the atrial myocardium and conduction system, as well as around coronary arteries, during the second trimester. Cell bodies in cardiac ganglia were found to be non-immunoreactive for VIP.

Nerves containing immunoreactivity for the putative sensory neuropeptides CGRP and SP became apparent only in the later stages of fetal development, i.e. at 18–24 wk of gestation. They exhibited a similar distribution pattern, occurring mainly in nerve trunks and fascicles associated with the great vessels and coronary arteries (Fig. 7) and between non-immunoreactive ganglion cells.

DISCUSSION

Previous studies have shown that neurons and nerves reach the developing heart in the late embryonic period (Navaratnam, 1965*a*; Smith, 1970, 1971; Gardner & O'Rahilly, 1976; Shvalev & Sosunov, 1989). In the present investigation a sensitive immunohistochemical method was used to demonstrate the presence of PGP 9.5 and neurofilament-immunoreactive neurons in the 7–8 wk embryonic heart, confirming the view that human cardiac innervation commences relatively early. At 14 wk of gestation, 5 primary groups of intracardiac ganglia can be identified, localised to the adventitia of the aorta and pulmonary artery, left and right atrial endocardium, the interatrial septum and the atrioventricular groove (Smith, 1970). Further evidence of the early development of human cardiac innervation was obtained by the immunohistochemical localisation of synaptophysin to varicose nerve fibres in the fetal heart, this protein being associated with the membrane of small secretory vesicles in nerve terminals (Wharton et al. 1990). These immunohistochemical findings are consistent with the ultrastructural demonstration of secretory vesicles and synapse formation in fetal cardiac ganglia at 8 to 10 wk of gestation (Shvalev & Sosunov, 1989). As in previous histological and histochemical studies (Navaratnam, 1965*a*; Smith, 1970; Kanerva et al. 1974; Gardner & O'Rahilly 1976), a progressive invasion of nerves was found in the fetal heart, occurring first in the atria and conduction system and then extending with the coronary vasculature into the ventricles at 12–14 wk of gestation. The localisation of N-CAM immunoreactivity to neural and extraneuronal structures within the heart of 7–24 wk of gestation is also consistent with the proposal that this cell surface sialoglycoprotein may play a part in the development of cardiac innervation (Gordon et al. 1990).

The ontogenesis of parasympathetic and intrinsic cardiac innervation is considered to precede that of sympathetic innervation in most mammals, including man (Pappano, 1977; Epstein, 1990). This view was prompted by the findings of morphological and histochemical studies showing that neurons and acetylcholinesterase-positive nerves are present in the fetal heart before catecholamine-containing cells can be demonstrated. Studies employing formaldehyde-induced fluorescence techniques to demonstrate catecholamine-containing nerves have found weak fluorescence staining in nerve trunks associated with coronary blood vessels at 12–15 wk of gestation and in intramyocardial nerves at 15–16 wk of gestation, whereas catecholamine-containing nerves were not identified in fetal hearts examined up to 18 wk of gestation (Dail & Palmer, 1973; Kanerva et al. 1974; Partanen & Korkala, 1974). Other investigators have found that the human fetal heart is capable of noradrenaline synthesis as early as the 13th wk of gestation (Gennser & von Stunditz, 1975), although this may not be indicative of extrinsic adrenergic innervation, as non-neuronal sources of catecholamine synthesis also exist in the fetal heart, with small intensely fluorescent paraganglion cells present from between the 8th and 10th wk of gestation (Dail & Palmer, 1973; Partanen & Korkala, 1974). In the present study tyrosine hydroxylase immunoreactivity was demonstrated in nerve trunks and paraganglion cells from 7 wk of gestation, and was also localised to intracardiac neurons and nerve fibres, neither of which have been found to display formaldehyde-induced fluorescence staining for catecholamines. The detection of the rate-limiting enzyme in catecholamine synthesis need not, however, correlate with catecholamine synthesis and sympathetic function (Pappano, 1977) and although intracardiac fetal neurons have been found to display 5-HT immunoreactivity in culture they do not appear to contain dopamine β-hydroxylase (Hassall et al. 1990). Furthermore, the

expression of tyrosine hydroxylase by intracardiac neurons seems to be a transient phenomenon as we have not detected cell bodies immunoreactive for this enzyme in either the infant, adolescent or adult human heart (Wharton et al. 1990). Thus it would appear that, during the development of the human cardiac innervation, intracardiac neurons undergo a phenotypic change and may give rise to some of the tyrosine hydroxylase-immunoreactive nerves identified in the early fetal heart.

NPY-immunoreactive varicose nerves were identified in the fetal heart at the 10th week of gestation. While the absence of immunostained neurons is consistent with the proposed extrinsic origin of these nerves in the human heart (Wharton et al. 1990) a minor subpopulation (< 1 %) of intracardiac neurons, cultured from human fetal atria, have been found to display immunoreactivity for both NPY and the C-terminal flanking peptide of its precursor sequence (Hassall et al. 1990). The paucity of this subpopulation may explain why similar neurons were not identified in situ, either in the fetal or adult human heart (Wharton et al. 1990). Alternatively the expression of NPY by intrinsic neurons may be a consequence of the culture conditions. In the adult human heart NPY-immunoreactive nerve fibres exhibit a similar distribution pattern to those displaying tyrosine hydroxylase immunoreactivity and are considered to represent sympathetic nerve terminals (Wharton et al. 1990). As such, the appearance of NPY-immunoreactive nerves in the fetal heart at 10 wk of gestation implies that cardiac adrenergic innervation commences at a relatively early stage, before that previously shown by formaldehyde-induced fluorescence staining of catecholamine-containing nerves (Dail & Palmer, 1973; Kanerva et al. 1974; Partanen & Korkala, 1974). This apparent discrepancy may reflect differences in either the sensitivity of the immunohistochemical and histochemical techniques used or the synthesis and storage of NPY and catecholamines in the developing cardiac innervation.

The appearance of somatostatin-immunoreactive nerve fibres between 10 and 12 wk of gestation coincided with the demonstration of cell bodies in cardiac ganglia displaying somatostatin immunofluorescence staining and suggests that this subpopulation of nerves is of intrinsic origin. This is consistent with the observation that most intracardiac neurons, cultured from human fetal hearts at 15–20 wk of gestation, display somatostatin immunoreactivity (Hassall et al. 1990), as well as with previous studies indicating that somatostatin-immunoreactive nerve fibres are an intrinsic component of the innervation of the adult heart (Day et al. 1985; Franco-Cereceda et al. 1986). Immunohistochemical studies on the innervation of the canine heart have suggested that VIP-immunoreactive nerves are also of intrinsic origin (Weihe & Reinecke, 1981; Weihe et al. 1984). This peptide-containing nerve population first appeared in the 10th wk of gestation and remained relatively sparse up to and including the 24th wk. The distribution of these nerve fibres and the absence of immunostaining in cardiac neurons corresponds with our previous findings (Wharton et al. 1990), indicating that VIP-immunoreactive nerves make a relatively modest contribution to the extrinsic innervation of the human heart.

Immunohistochemical studies using the sensory neurotoxin capsaicin have established that CGRP and tachykinins such as SP occur together in afferent nerves supplying the mammalian cardiovascular system (Wharton & Gulbenkian, 1987). We have previously shown that CGRP and SP immunoreactivities are colocalised in nerve fibres in the adult human heart although, in comparison with the rat and guinea pig, they are relatively few in number (Wharton et al. 1986, 1988, 1990). In the developing fetal heart putative sensory nerve fibres containing CGRP and SP immunoreactivity exhibited a similar distribution pattern to that observed in the adult, localised in nerve trunks and around non-immunoreactive intracardiac neurons.

The appearance of these immunostained nerve fibres at 18–24 wk of gestation was a relatively late event compared with that of other peptide-containing cardiac nerves and suggests that the onset of the sensory component follows that of the autonomic innervation. The relatively late onset of the CGRP-immunoreactive innervation in the human heart is similar to that observed in the dog (Ursell et al. 1991), although the perinatal peak in neural density recorded in the canine heart could not be confirmed in the present study due to limitations of available tissue.

Although there is some evidence suggesting neural modulation of fetal heart rate (Fine et al. 1988) and adrenergic neurotransmission in fetal atria (Walker, 1975) the presence of intracardiac neurons and peptide-containing nerves may not reflect functional innervation of the developing heart (Pappano, 1977; Epstein, 1990). Peptides such as somatostatin have, however, been considered to influence organ maturation and neural phenotypic expression (see Hassall et al. 1990) and the developing innervation may modulate the differentiation of smooth muscle cells (Chamley et al. 1974) and cardiac myocytes (Kohtz et al. 1989).

In summary, the development of peptide-containing nerves has been examined in an extensive series of specimens of human fetal heart, ranging from the late embryonic period through to the end of the 6th month of gestation. Immunoreactivity for NPY and the catecholamine-synthesising enzyme tyrosine hydroxylase was detected in cardiac nerve fibres relatively early in gestation, before the adrenergic innervation is considered to be established. The onset of the CGRP- and SP-immunoreactive innervation was preceded by the appearance of other peptide-containing nerves, suggesting that afferent innervation occurs later than the autonomic innervation. While somatostatin-immunoreactive nerve fibres may be of intrinsic origin, the other peptide-containing nerve subpopulations identified appear to be of extrinsic origin. Whether these peptides influence fetal cardiac function or have a trophic role, modulating the maturation of cardiac innervation and nonneuronal cells in the developing heart, remains to be established.

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