Immunohistochemical Demonstration of Human Cardiac Innervation Before and After Transplantation

John Wharton, Julia M. Polak, Lee Gordon, Nicholas R. Banner, David R. Springall, Marlene Rose, Asghar Khagani, John Wallwork, and Magdi H. Yacoub

Knowledge about the distribution and origins of peptide-containing nerves in the innervated and transplanted heart is lacking. Immunohistochemical and histochemical techniques were used to visualize human cardiac innervation before and after transplantation. In the recipient heart cardiac nerve fibers and fascicles displayed immunoreactivity for general neural (protein gene product 9.5 and synaptophysin) and Schwann cell markers (S-100). A major proportion of cardiac nerves displayed neuropeptide tyrosine and tyrosine hydroxylase immunofluorescence staining. Subpopulations of nerves contained somatostatin, vasoactive intestinal polypeptide, calcitonin gene-related peptide, substance P- or neurokinin-like immunoreactivity, and acetylcholinesterase activity. Tissues from cardiac allografts (5 weeks to 63 months after transplantation) contained nerves and ganglion cells that were acetylcholinesterase positive and immunoreactive for the general neural markers. These nerves were less numerous than in recipient hearts and rarely displayed neuropeptide immunostaining. Atrial natriuretic peptide immunoreactivity was localized to myocardial cells in transplanted hearts as well as explanted recipient and postmortem hearts. While most human cardiac allografts remain functionally extrinsically denervated, they appear to contain viable intrinsic nerves, and myocardial cells retain the capacity to produce atrial natriuretic peptide. (Circulation Research 1990;66:900–912)

Cardiac innervation may now be visualized by use of sensitive immunohistochemical methods and antisera to neural marker proteins such as protein gene product 9.5 and synaptophysin.1–3 Efferent and afferent nerves may also be identified on the basis of their enzymatic or transmitter content, and several biologically active neuropeptides have been found in different subpopulations of mammalian cardiovascular nerves.2 However, there are considerable species variations in the number and distribution of nerve subtypes in the mammalian heart. In the guinea pig heart, for example, there are many putative sensory nerves that display tachykinin and calcitonin gene-related peptide immunoreactivity, whereas in other mammals these nerves occur relatively infrequently. It is uncertain to what extent peptide-containing nerves represent neurons extrinsic or intrinsic to the heart, and there is also limited information regarding the distribution of these nerves in the human heart.2,3

Cardiac tissues receive both efferent sympathetic and parasympathetic nerves and an afferent innervation.2 During heart-lung transplantation, the extrinsic nerves supplying the donor heart are sectioned, resulting in both efferent and afferent decentralization.3–10 The presence of nerves and ganglion cells has been noted in tissues obtained postmortem from human cardiac allografts by use of conventional histochemical techniques.8,11,12 Little is known about the nature of these nerves, and in contrast to experimental animals, functional reinnervation has not generally been observed in long-term human transplant patients.5,8,9,13

The aims of the present study were to compare the innervation of the human heart before and after transplantation, define the distribution of peptide-containing nerves, and gain insight into their intrinsic or extrinsic origins. In addition, the presence of atrial natriuretic peptide immunoreactivity in allograft myocardium was examined.

From the Department of Histochemistry, Royal Postgraduate Medical School, London; Cardiothoracic Unit (N.R.B., M.R., A.K., M.H.Y.), Harefield Hospital, Harefield, Middlesex; and Transplant Unit (J.W.), Papworth Hospital, Papworth Everard, Cambridge, U.K.

Supported in part by the British Heart Foundation.

Address for reprints: John Wharton, PhD, Department of Histochemistry, Royal Postgraduate Medical School, Du Cane Road, London W12 ONN, UK.

Received April 24, 1989; accepted October 13, 1989.
Table 1. Clinical Features of Patients Receiving a Primary Cardiac Transplant

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Type of transplant</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>2</td>
<td>H/L</td>
<td>Tricuspid atresia</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>1</td>
<td>H/L</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>59</td>
<td>HT</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>59</td>
<td>HT</td>
<td>Dilated cardiomyopathy*</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>28</td>
<td>HT</td>
<td>Adriamycin cardiomyopathy</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>6</td>
<td>H/L</td>
<td>Eisenmenger syndrome</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>28</td>
<td>H/L</td>
<td>Idiopathic pulmonary hemosiderosis</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>29</td>
<td>H/L</td>
<td>Sarcoïdosis</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>21</td>
<td>H/L</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>14</td>
<td>H/L</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>25</td>
<td>H/L</td>
<td>Cystic fibrosis</td>
</tr>
</tbody>
</table>

HT, heart transplant; H/L, heart and lung transplant.
*Predominantly affecting the right side.

Materials and Methods

Cardiac samples were obtained from 20 patients (aged 1–59 years; mean±SEM, 25.2±3.8 years) during heart and lung transplantation. Recipient tissues were obtained from 11 patients (aged 1–59 years; 24.8±6 years) undergoing primary heart or heart-lung transplantation (Table 1). Allograft and recipient tissues were also obtained from an additional nine patients (aged 3–48 years; 25.7±4.9 years) undergoing a second transplant procedure (Table 2). In the latter group, seven patients had received a primary heart and lung allograft and two had received a heterotopic heart transplant. The period between the primary and secondary transplant procedures ranged from 5 weeks to 63 months (Table 2). A further five hearts, from patients without cardiac complications (aged 24–75 years; 51.0±10.0 years), were examined at postmortem after a 3- to 15-hour delay between death and sampling. The collection of these tissues followed the ethical standards of the institutions in which they were obtained. Heterotopic cardiac transplantation was performed as previously described. The operative technique for heart-lung transplantation has also been described and results in extrinsic denervation of both the donor heart and lungs.15

Tissue Preparation

Cardiac tissues were processed immediately after surgical removal. Full-thickness transmural samples were taken from the lateral walls of both ventricles, the interventricular septum, papillary muscles, and both atria. In some cases, atrial trimmings were obtained from donor hearts, and the site of anastomosis between the recipient and primary donor aorta or pulmonary artery was also sampled. All tissues were fixed by immersion for approximately 16 hours at 4°C in a modified Bouin’s solution containing 85 ml of 2% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) and 15 ml of saturated picric acid per 100 ml of fixative. After the tissues were rinsed in several changes of phosphate buffered saline (0.01 M, pH 7.2) containing 15% sucrose and 0.1% sodium azide, cryostat blocks were prepared by orientating the tissue on a cork mat, surrounding it in mounting medium (Tissue-Tek, Miles Inc, Elkhart, Indiana), and rapidly freezing it in melting dichlo-

Table 2. Clinical Features of Retransplant Patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Diagnosis</th>
<th>Type</th>
<th>Donor sex</th>
<th>Age (years)</th>
<th>Ischemia time (minutes)</th>
<th>Duration (months)</th>
<th>Secondary transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>10</td>
<td>PF</td>
<td>H/L</td>
<td>M</td>
<td>5</td>
<td>220</td>
<td>1</td>
<td>Rejection</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>25</td>
<td>CF</td>
<td>H/L</td>
<td>M</td>
<td>8</td>
<td>180</td>
<td>8</td>
<td>Recurrent infection</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>18</td>
<td>PPH</td>
<td>H/L</td>
<td>F</td>
<td>17</td>
<td>90</td>
<td>10</td>
<td>OB</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>18</td>
<td>PPH</td>
<td>H/L</td>
<td>F</td>
<td>20</td>
<td>175</td>
<td>11</td>
<td>OB</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>9</td>
<td>CONG+PH</td>
<td>H/L</td>
<td>F</td>
<td>7</td>
<td>215</td>
<td>13</td>
<td>OB</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>33</td>
<td>PPH</td>
<td>H/L</td>
<td>F</td>
<td>13</td>
<td>100</td>
<td>16</td>
<td>OB</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>3</td>
<td>PPH</td>
<td>H/L</td>
<td>F</td>
<td>2.5</td>
<td>160</td>
<td>21</td>
<td>OB</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>43</td>
<td>IHD</td>
<td>HT (Het)</td>
<td>M</td>
<td>15</td>
<td>102</td>
<td>46</td>
<td>Rejection</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>48</td>
<td>IHD</td>
<td>HT (Het)</td>
<td>M</td>
<td>20</td>
<td>195</td>
<td>63</td>
<td>Rejection</td>
</tr>
</tbody>
</table>

PF, pulmonary fibrosis; CF, cystic fibrosis; PPH, primary pulmonary hypertension; CONG+PH, congenital heart defect and pulmonary hypertension; IHD, ischemic heart disease; H/L, heart and lung transplant; HT (Het), heart transplant (heterotopic); OB, obliterative bronchiolitis.
rodifluoromethane (Arcton, ICI, Cheshire, UK) suspended in liquid nitrogen.

**Immunofluorescence Staining**

An indirect immunofluorescence staining procedure and specific primary antisera were employed for investigation of the innervation of the tissues. The origins and specificity of these antisera have been previously described. Briefly, sections 15 μm thick were cut in a cryostat (Bright, Huntington, UK) at −25°C, mounted on poly-L-lysine-coated slides, and air-dried for 1 hour at room temperature. After immersion in buffered saline containing 0.2% Triton X-100 for 30 minutes and rinsing in buffer, the sections were stained with pontamine sky blue (BDH, Poole, UK) for 30 minutes for reduction of background fluorescence. Sections were then rinsed in buffer, incubated with diluted primary antisera for 16–24 hours at 4°C, rinsed again, and incubated with fluorescein isothiocyanate–labeled sheep anti-rabbit IgG (diluted 1:100; Wellcome Diagnostics, Dartford, UK) for 1 hour at room temperature. After further rinsing the preparations were mounted in glycerol mixed 2:1 with buffered saline and examined with a microscope (model AH-2, Olympus, Lake Success, New York) equipped for epi-illumination. Controls included the omission of the primary antiserum, replacement of the primary antiserum with preimmune serum, and preabsorbance of the antisera with their respective antigens (10⁻⁵–10⁻¹⁰ M).

The relative number of different immunostained nerves, in both the innervated and transplanted heart, was visually assessed in a semi-quantitative manner and arbitrarily graded from 0 to ++++. Table 3. Examples of this grading are given in the figures. The relative number of immunostained nerves in the perivascular plexus around coronary arteries and arterioles was also related to vessel location and size by means of measurements taken from 35-mm photographic negatives of immunostained preparations by use of a ×10 eyepiece micrometer. An average value of external diameter at the adventitial-medial border was obtained for each transversely sectioned vessel from two measurements made at right angles to one another.

**Acetylcholinesterase Staining**

The localization of acetylcholinesterase activity was investigated in alternate sections and in immu-

**Results**

**Immunohistochemistry and Histochemistry of Innervated Hearts**

Cardiac innervation was demonstrated by use of immunofluorescence techniques and antisera to general neural markers, neurofilament proteins, protein gene product 9.5, and synaptophysin. Immunoreactive nerve fibers and fascicles were distributed throughout the atrial and ventricular walls and exhibited a gradient in the relative number of nerve fibers from the atria to the ventricles (Table 3). Varicose nerve fibers were distributed among myocardial cells, around branches of coronary vessels, and in an endocardial plexus (Figure 1). In the tissue samples examined, intrinsic cardiac ganglia were confined to the atrial subepicardium and atroventricular groove; none were found in ventricular tissues (Figure 2). An inverse relation was observed between coronary artery external diameter and the number of perivascular nerves (Table 3). Epicardial and large intramyocardial coronary arteries (0.4–3.0 mm external diameter) were accompanied by nerve trunks but received relatively few nerve fibers at the adventitial-medial border (Figure 2). More distal artery branches (100–400 μm external diameter) possessed a moderate nerve supply. The perivascular plexus was concentrated around small intramyocardial arteries and arterioles (25–100 μm external diameter), where numerous immunoreactive nerves were found to encompass the vessels at the adventitial-medial border (Figures 2 and 3).

Many nerve fibers and fascicles displayed neuropeptide tyrosine immunoreactivity; they were concentrated in the myocardium, endocardium, and around the smaller branches of coronary arteries (Figures 2 and 3). Tyrosine hydroxylase-immunoreactive nerves exhibited a similar distribution pattern. Subpopulations of nerve fibers possessed immunoreactivity for other neuropeptides, the most common being somatostatin, which was localized to nerve fibers concentrated in atrial myocardium and endocardium and around intramyocardial arteries (Figures 4a and 4b). Nerve fibers displaying vasoactive intestinal polypeptide immunoreactivity had a similar distribution pattern, while those containing calcitonin gene-related peptide, substance P, or neuropeptide Y immunoreactivity were relatively sparse, occurring mainly around neural cell bodies in intrinsic ganglia and in nerve trunks (Figures 4c–g; Table 3). No evidence was obtained of peptide immunoreactivity occurring in intrinsic neural cell bodies, although peptide-containing nerve fibers were regularly identified in cardiac ganglia (Figures 4e–g). The relative rank order of peptide-containing cardiac nerves identified in the heart tissues was neuropeptide Y > somatostatin > vasoactive intestinal polypeptide > calcitonin gene-related peptide, substance P, and neuropeptides (Table 3).

There was a general trend for the number of immunostained nerves to decline in all regions with age. This decrease was associated with an increase in fibroelastic tissue. In cases exhibiting endocardial thickening and fibrosis, the endocardial plexus appeared to contain fewer nerves and was often confined to regions immediately below the endothelial cell layers or toward the subendocardial-myocardial junction rather than distributed throughout the endocardium. Scared areas of infarcted myocardium also lacked immunoreactive nerves. The overall pattern of innervation and relative number of nerves were similar in the postmortem and surgical cases.

Acetylcholinesterase activity was demonstrated in intrinsic neural cell bodies and in many of the nerves displaying protein gene product 9.5 immunoreactivity (Figure 3). Due to the limited resolution of the light microscope, it was not possible to distinguish individual axons possessing cholinesterase activity or peptide immunoreactivity, but both populations appeared to be less numerous than total innervation identified with primary antisera to protein gene product 9.5.

Atrial natriuretic peptide immunoreactivity was localized to myocardial cells throughout the right and left atrial walls, in the ventricular conduction system, and in the ventricular myocardium of the failing explanted heart. The ventricular myocardium of postmortem cases without cardiac complications did not display immunostaining with antisera to atrial natriuretic peptide.

**Immunocytochemistry and Histochemistry of Transplanted Hearts**

Atrial and ventricular tissues from all nine cardiac allografts (5 weeks to 63 months after implantation) contained nerve fibers and fascicles displaying neurofilament, protein gene product 9.5, or synaptophysin immunoreactivity (Figure 5). The relative number of immunostained nerves was less than in recipient hearts of similar age that possessed an intact innervation. An atrial-to-ventricular gradient was observed in the number of immunoreactive nerves demonstrated in the allografts (Table 3).
Nerve fibers in ventricular allograft tissues were usually sparse, fine, and varicose; exhibited protein gene product 9.5 immunoreactivity; and were accompanied by S-100-immunoreactive Schwann cells (Figure 5b). Scattered ganglia and ganglion cells were observed in the atrial tissues displaying immunoreactivity for general neural markers (Figure 5c). Nerve trunks identified in the epicardium and myocardium contained immunoreactive fibers that sometimes appeared swollen and fragmented, while in other regions fine varicose immunoreactive fibers were observed extending out from nerve trunks (Figures 5d and 5e). The donor aorta and pulmonary and coronary arteries exhibited a general loss of nerves in the adventitia and around the vasa vasorum (Figures 6a and 6b). A concentric intimal proliferation of coronary arteries and their branches was observed in four of the nine allografts.

A notable feature of the neural immunostaining in the cardiac allograft tissues was the lack of neuropeptide tyrosine- and tyrosine hydroxylase-immunoreactive nerves (Figures 6c–e). This finding was in contrast to recipient hearts, in which these nerves predominated (Table 3). These nerves were either absent altogether or were rarely detected, as were atrial fibers displaying vasoactive intestinal polypeptide, somatostatin, calcitonin gene-related peptide, and substance P immunoreactivity. In addition to displaying protein gene product 9.5 and neurofilament immunoreactivity, nerve fibers in the allograft...
tissues contained synaptophysin, and most appeared to possess acetylcholinesterase activity (Figures 6f and 6g). Neuronal cell bodies contained acetylcholinesterase activity, but lacked neuropeptide immunoreactivity. Synaptophysin- and peptide-immunoreactive nerve fibers were rarely detected in ganglia in allograft tissues (Figures 7a–c). The number and distribution of nerves in the cardiac allografts showed no apparent correlation with ischemic time, duration of graft, rejection, or the position in which the graft was placed.

Atrial natriuretic peptide immunoreactivity was localized to myocardial cells throughout the atria of allografts obtained 5 to 63 months after either heterotopic heart or combined heart and lung transplantation (Figures 7f and 7g). Ventricular subendocardial conducting and myocardial cells displayed immunoreactivity as well, but unlike explanted recipient hearts, some allografts also exhibited a subepicardial immunostaining of the ventricular myocardium.

Control Sections

No immunofluorescence staining was obtained when primary antisera were omitted, replaced with preimmune serum, or preabsorbed with their respective antigens. All the antisera were found to be specific except for the substance P antiserum, which exhibited a partial cross-reactivity with other tachykinins (neurokinin A and B). Specific acetylcholinesterase activity was not detected when acetylthiocholine iodide was omitted or replaced with butyrylthiocholine iodide and tetraisopropyl pyrophosphoramide (10^-4 to 10^-6 M) was added. The addition of eserine (10^-4 M) to the incubation medium also inhibited the development of a positive reaction.

Discussion

The majority of pretransplant tissues were obtained from failing explanted hearts, but postmortem and transplant donor tissues without intrinsic heart disease provided similar results, indicating that end-stage cardiac failure did not significantly alter the relative numbers of immunoreactive nerves. However, pronounced changes were observed in the innervation of cardiac tissues after cardiac transplantation; there were fewer nerves in general and a loss of peptide-containing nerves in particular.

Neuropeptide Tyrosine–Containing Nerves

Neuropeptide tyrosine–immunoreactive nerves represented the most numerous population of peptide-containing nerves identified in the human heart and had an extensive distribution pattern, which was similar to that of nerves containing tyrosine hydroxylase. The loss of these nerves in cardiac allografts indicates that they are of extrinsic origin. This conclusion is concordant with the effects of
surgical and chemical cardiac sympathectomy in experimental animals, which also results in a loss of immunoreactive nerves as well as a significant reduction (50%-90%) in the tissue levels of both neuropeptide tyrosine and noradrenaline.22–27 It seems likely that the majority of neuropeptide tyrosine–immunoreactive nerves in the mammalian heart represent postganglionic sympathetic neurons originating in the stellate and other paravertebral ganglia, where numerous neuropeptide tyrosine–containing cell bodies have been identified.22,23,28 Postganglionic sympathetic cardiac nerves arise from the upper thoracic and cervical paravertebral ganglia, but the principal site of synapses between preganglionic and postganglionic sympathetic neurons varies with species and is uncertain in man.4 As previously reported, neuropeptide tyrosine–immunoreactive cardiac ganglion cells were not detected in the human heart.28 This finding contrasts with data obtained in the rat heart, where some ganglion cells have been found to display immunoreactivity26,29,30 and may contain ribonucleic acid transcripts encoding the synthesis of the neuropeptide tyrosine precursor molecule.31,32 The extent to which the peptide occurs in classical postganglionic sympathetic or intrinsic cardiac neurons probably exhibits species variation. While

**Figure 3.** Immunofluorescence micrographs of serial sections of right atrium from an explanted heart showing myocardial and perivascular nerves displaying immunoreactivity for protein gene product 9.5 (PGP), C-terminal flanking peptide of neuropeptide Y (C-PON), and tyrosine hydroxylase (TH) and acetylcholinesterase (AChE) activity. Many nerves show PGP immunoreactivity (panel a; +++++). A large number also display C-PON (panel b; ++++) and TH immunoreactivity (panel c; ++++) or AChE activity (panel d; ++++) and are concentrated in perivascular plexus around small arteries and arterioles (A, 75 μm external diameter) as well as among atrial myocardial fibers. Bar=50 μm.
FIGURE 4. Immunofluorescence micrographs of sections of left atrium from an explanted heart immunostained for the C-terminal flanking peptide of neuropeptide Y (C-PON), somatostatin (SOM), calcitonin gene-related peptide (CGRP), and vasoactive intestinal polypeptide (VIP). Compared with the large number of myocardial and perivascular nerves displaying C-PON immunoreactivity (panels a and c; +++), there are a moderate number of SOM-containing nerves (panel b; ++) and relatively few CGRP-immunoreactive fibers (panel d; arrows; +). Panels a/b and c/d are of serial sections. The only CGRP-immunoreactive nerve fibers identified in the whole field of panel c occurred in perivascular plexus (inset, panel d). Panels e through g demonstrate C-PON-, VIP-, and CGRP-immunoreactive varicose nerve fibers distributed among nonreactive neuronal cell bodies in a subepicardial atrial ganglion. Bar=50 μm.
intrinsic neurons may make an important contribution in the rat, they probably do not represent a significant source of neuropeptide tyrosine-containing nerves in human heart. It is possible, however, that some loss of immunoreactivity reflects a disruption of the cardiac ganglia secondary to transplantation.

Sensory Peptide–Containing Nerves

Immunohistochemical studies employing the sensory neurotoxin capsaicin have established that calcitonin gene-related peptide and tachykinins such as substance P occur together in afferent nerves supplying the mammalian cardiovascular system. We have also demonstrated that both peptides occur
**FIGURE 6.** Immunofluorescence micrographs of sections of aortic wall (panels a and b), right ventricle (panels c through e), and atrial septum (panels f and g) 11 months (a and b), 21 months (c through e), and 10 months (f and g) after heart and lung transplantation. Protein gene product 9.5 (PGP)-immunoreactive perivascular nerves occur around small arteries and arterioles (A) in adventitia proximal (panel a; ++++) but not distal (panel b; o) to aortic anastomosis. Elastic lamina in media of aorta (Ao) display fluorescence due to pontamine sky blue counterstain. In serial sections of allograft right ventricle, PGP-immunoreactive nerves are present in myocardium and endocardium (panel c; + +); however, they lack immunoreactivity for C-terminal flanking peptide of neuropeptide Y (panel d, C-PON; o) and tyrosine hydroxylase (panel e, TH; o). Sequential immunofluorescence staining for PGP (panel f) and acetylcholinesterase (AChE) staining (panel g), on same section of atrial septum, demonstrate that PGP-immunoreactive nerves present in allograft also possess AChE activity. Bar=50 μm.
FIGURE 7. Immunofluorescence micrographs of sections of left atrium (panels a, b, and d) and right ventricle (panel e) 63 months after heterotopic heart transplantation and right atrium (panel c) 13 months after combined heart and lung transplantation. Only rare varicose fibers (arrows) in intrinsic ganglia (panels a and b; ±) and myocardium (panel c; ±) display immunoreactivity for synaptophysin (SYN) and C-terminal flanking peptide of neuropeptide Y (C-PON) after cardiac transplantation. ANP-immunoreactive myocardial cells are demonstrated in left atrium (panel d) and right ventricle (panel e) of an allograft 63 months after heterotopic heart transplantation; immunoreactivity displays a characteristic perinuclear localization (arrows). Bar=50 μm.

Together in nerve fibers in the human atrial appendage.\(^3\) However, in comparison with other animals such as the guinea pig, nerve fibers containing either calcitonin gene-related peptide or substance P immunoreactivity occur relatively infrequently in the human heart. Nerve fibers displaying immunoreactivity for both peptides were characteristically found in nerve trunks and around intrinsic neuronal cell bodies in cardiac ganglia, suggesting a possible interaction between afferent nerves and the autonomic innervation of the heart. The apparent absence of calcitonin gene-related peptide and substance P-immunoreactive nerve fibers in the cardiac allografts is consistent with an extrinsic origin for these nerves, presumably representing primary sensory vagal or spinal neurons.

**Somatostatin and Vasoactive Intestinal Polypeptide–Containing Nerves**

Somatostatin-immunoreactive nerves had a distribution pattern distinct from those displaying immunoreactivity for neuropeptide tyrosine, calcitonin gene-related peptide, or substance P, and were concentrated in atrial rather than ventricular tissues. This distribution pattern corresponds to that of parasympathetic nerves,\(^4\) and the finding of somatostatin-immunoreactive neuronal cell bodies in the developing\(^3\) and adult human heart has led to the suggestion that somatostatin-containing nerves represent intrinsic, postganglionic parasympathetic neurons.\(^28\) An extrinsic origin cannot be excluded in view of the apparent loss of immunostained nerve fibers after transplantation, but it is also possible that the absence of a functional preganglionic innervation results in the down-regulation of peptide expression in postganglionic cardiac neurons. The apparent loss of somatostatin-immunoreactive nerves could also reflect a disruption of intrinsic cardiac ganglia secondary to transplantation. Nerves displaying vasoactive intestinal polypeptide immunoreactivity were also concentrated in the atria; however, they appeared to be less numerous than those containing somatostatin. The origin of these nerves is uncertain, for while vasoactive intestinal polypeptide–immunoreactive ganglion cells have been described in the dog,\(^38\) they were not demonstrated in the human heart.
Cardiac Allografts

Nerve fibers, fascicles, and ganglion cells demonstrated in cardiac allografts were concentrated in atrial tissue, displayed immunoreactivity for general neural markers, and possessed acetylcholinesterase activity. The persistent functional denervation of the transplanted human heart\textsuperscript{5,6,8,9} suggests that these nerves represent intrinsic postganglionic neurons. The functional status of the neurons is not known, but the localization of synaptophysin immunoreactivity in varicose nerve fibers indicates that they possess secretory vesicles of the type that are likely to contain classical neurotransmitters such as acetylcholine.\textsuperscript{39}

In contrast to humans, the transplanted hearts of experimental animals have been shown to exhibit functional extrinsic reinnervation.\textsuperscript{6,40} A gradual increase in tissue catecholamine levels and catecholamine-containing nerves parallels the return of autonomic responses,\textsuperscript{7,41,42} and this increase may be enhanced by treatment with nerve growth factor.\textsuperscript{43} If reinnervation can occur in humans, it would appear to require a much longer time than in experimental animals and could be hindered by rejection. The distinction between reinnervation in humans and animals may represent either a species difference or variations between allografts and autografts since may of the animal experiments involved autotransplantation. However, evidence has been obtained suggesting that functional reinnervation may occur in a minority of heterotopic allografts,\textsuperscript{44} and a recent study identified one case out of nine in which electrocardiographic and indirect clinical evidence of functional reinnervation was obtained 33 months after orthotopic cardiac transplantation.\textsuperscript{45}

Atrial Natriuretic Peptide

The distribution pattern of atrial natriuretic peptide immunoreactivity in the cardiac allografts was comparable, with that found in failing explanted human hearts,\textsuperscript{17} indicating that myocardial cells retain the capacity to produce the peptide for at least up to 5 years after transplantation and that this capacity is not dependent on a functional autonomic innervation. Basal plasma levels are raised in cardiac transplant patients, but the atrial natriuretic peptide secretory and hemodynamic responses to lower body positive pressure do not appear to be impaired by extrinsic cardiac denervation.\textsuperscript{46,47}

In conclusion, nerves containing peptides such as neuropeptide tyrosine are prominent in the human heart, but are depleted in cardiac allografts and probably represent extrinsic neurons. The transplanted human heart appears to remain extrinsically denervated, but retains an intrinsic nerve supply and the capacity to produce atrial natriuretic peptide.

Acknowledgments

Antisera raised to regulatory peptides at Hammersmith Hospital, London, were produced in conjunction with Professor S.R. Bloom. Antisera to synaptophysin and neurofilaments were generously provided by Dr. R. Jahn and Dr. D. Dahl, respectively. We are grateful to Professor S.G. Haworth, Great Ormond Street Hospital for Sick Children, London, for providing samples from one of the primary transplant cases. The authors wish to thank the transplant coordinator and staff of the Immunology Department, Harefield Hospital, for their assistance and Patricia Harley for excellent technical assistance.

References


23. Lundberg JM, Saria A, Franco-Cereceda A, Hokfelt T, Tere-

nius L, Goldstein M: Differential effects of reserpine and 6-hydroxydopamine on neuropeptide Y (NPY) and noradrenergic neuropeptides in the peripheral and central nervous system. Naunyn Schmiedebergs Arch Pharmacol 1985; 328:331–340


27. Maccarrone C, Jarrott B: Differential effects of surgical sympa-


30. Hassall CIS, Burnstock G: Neuropeptide Y–like immunoreac-


38. Weihe E, Reinecke M, Forsmann WG: Distribution of vaso-


45. Fallen EL, Kamath MV, Ghista DN, Fichett D: Spectral analysis of heart rate variability following human heart transplanta-


KEY WORDS • cardiac innervation • transplanted heart • immunohistochemistry • extrinsic denervation • neuropeptides
Immunohistochemical demonstration of human cardiac innervation before and after transplantation.

J Wharton, J M Polak, L Gordon, N R Banner, D R Springall, M Rose, A Khagani, J Wallwork and M H Yacoub

doi: 10.1161/01.RES.66.4.900

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/66/4/900

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org//subscriptions/