
NORIO SATO

Obstetrics and Gynecology, Kurume University School of Medicine, Kurume 830-0011, Japan

Summary: The distribution of nerve fibers positive for calcitonin gene-related peptide (CGRP), which is a vasodilator peptide, neuropeptide Y (NPY), which is a vasoconstrictor peptide, and tyrosine hydroxylase (TH), which is a key enzyme for the synthesis of the neurotransmitter of adrenergic nerves, was studied in the fetal side, middle part, and placental side of the human umbilical cord using immunohistochemistry. In the fetal side of the umbilical cord, CGRP-, NPY-, and TH-positive fibers were observed in the smooth muscle of the media of the umbilical artery and in the margins of the Wharton jelly. They were not observed around the umbilical vein or in the middle part or placental side of the umbilical cord. These results demonstrate the presence of the vasoactive peptides CGRP and NPY, as well as of the enzyme TH in the fetal side of the umbilical vessel. The presence of CGRP and NPY suggests the involvement of these peptides in the regulation of the umbilical and placental circulation.

Key words umbilical cord, calcitonin gene-related peptide, neuropeptide Y, tyrosine hydroxylase

INTRODUCTION

The umbilical cord develops from the mesoderm in an early period of embryonic development, and is encapsulated by the amniotic membrane. Although the umbilical cord is considered to be involved in the maintenance of the fetoplacental circulation after the establishment of the placental circulation, little is known about its circulation-regulating functions.

Although the nerve distribution of the umbilical cord has been controversial, Matsubara and Tamada [1] demonstrated the presence of acetylcholine-esterase-positive fibers in part of the umbilical vessels by electron microscopy in 1988. Kawano and Mori [2] then demonstrated the presence of catecholamines in the fetal end of the umbilical cord by immunofluorescent staining and reported the presence of noradrenaline-containing sympathetic nerves. However, these investigations did not go beyond the demonstration of autonomic nerves such as the classic cholinergic and noradrenergic fibers.

These investigations also suggested that the umbilical vessels are subject to neural regulation, but few studies have supported this possibility.

Recently, autonomic nerves containing vasoactive peptides have been shown to participate in the vascular innervation. We, therefore, examined the presence of nerves containing vasoactive peptides in the human umbilical cord.

Calcitonin gene-related peptide (CGRP), which is a vasodilator peptide widely distributed in the cardiovascular system, neuropeptide Y (NPY), a vasoconstrictor peptide, and tyrosine hydroxylase (TH) were used for enzyme antibody and fluorescent antibody studies.

MATERIALS AND METHODS

The umbilical cords of term normal neonates were used. Each umbilical cord was divided into three portions: the fetal side, the middle and the placental side (Fig. 1).
The distribution of CGRP-, NPY-, and TH-positive nerve fibers was studied using immunohistochemical techniques.

**Immunohistochemistry**

Transverse cross-sections about 5 mm thick were cut from umbilical cords collected immediately after delivery and fixed in a 4% paraformaldehyde solution for 24 hs (4°C). The specimens were washed 3 times with phosphate buffer, pH 7.4, and dehydrated at 4°C twice with 7.5% sucrose for 3 hs, 10% sucrose for 12 hs, and 15% sucrose for 12 hs. Each specimen was frozen at −80°C and frozen sections 15 μm thick were cut on a cryostat. The sections were air-dried, and immersed in 0.01 M phosphate buffered saline (PBS) containing 0.3% Triton X-100 for 24 hs (4°C).

**Avidin-biotin complex method:** The sections were treated with PBS containing 1% H2O2 for 30 min to 1 h to avoid non-specific reaction. They were then treated with the primary antibody (Table 1) for 48 hs (4°C), washed with PBS, treated with biotin-labeled secondary antibody (anti-rabbit Ig) at 1:200 dilution for 2 hs (room temperature) and with HPR-labeled avidin-biotin complex (ABC) at 1:200 dilution for 2 hs, washed with PBS, treated with 3,3-diaminobenzidine (DAB) for 5-10 min for color reaction, and examined by light microscopy.

**TABLE 1. Immunohistochemistry**

<table>
<thead>
<tr>
<th>Fixation</th>
<th>Cut into frozen sections after cryoprotection</th>
<th>Sections washed with 0.01 M phosphate saline buffer with 0.3% Triton X-100 for 24 hrs (4°C)</th>
<th>Immunofluorescence (Texas Red labeled antibody)</th>
<th>ABC Method</th>
</tr>
</thead>
</table>

**TABLE 2. Results**

<table>
<thead>
<tr>
<th>Umbilical vessels</th>
<th>CGRP</th>
<th>NPY</th>
<th>TH</th>
</tr>
</thead>
<tbody>
<tr>
<td>F Artery</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M Artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P Artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vein</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*: positive  -: negative
F: fetal side  M: middle  P: placental side

Nerve fibers, immunoreactive to CGRP, NPY and TH, were observed in the fetal side of the umbilical cord, but not in the middle and in the placental side. In the fetal side of the umbilical cord, CGRP-, NPY- and TH-positive nerve fibers were present in the whole surroundings of the umbilical artery, showing an intimate association with the tunica media of the artery, but were absent umbilical vein.

Fig. 1. Ten term human umbilical cords were obtained immediately after delivery; each umbilical cord was divided into three portions: the fetal side, the middle, the placental side.
Fluorescent antibody method: The sections were treated with the primary antibody (Table 1) for 48 hs (4 °C), then with Texas Red-labeled secondary antibody (anti-rabbit Ig) at 1:200 dilution for 2 hs (room temperature), washed with PBS, sealed in glycerin, and observed by fluorescence microscopy.

Control experiments: To examine the specificity of each antibody, some sections were treated (1) without the primary antibody, (2) without the secondary antibody, (3) without ABC, and (4) the rat intestine was treated with the procedures described in 1), 2), and (1)-(3).

RESULTS

In the fetal side of the umbilical cord, NPY-positive fibers meandered with a bead-like appearance on the outer surface of the smooth muscle layer of the umbilical artery (Fig. 2a). The fluorescence antibody study showed localization of NPY-positive fibers in the smooth muscle layer of the umbilical artery (Fig. 2b). In the middle part or the placental side of the umbilicus, however, no NPY-positive fibers were detected in the umbilical artery or vein or the Wharton jelly. NPY-positive fibers were noted also on the margins of the Wharton jelly (Fig. 2c).

In the fetal side of the umbilical cord, TH-positive fibers were localized on the outer surface of the smooth muscle layer of the umbilical artery (Fig. 3a). TH-positive fibers were also noted on the margins of the Wharton jelly. The fluorescent antibody study confirmed the localization of TH-positive fibers on the outer surface of the smooth muscle layer of the umbilical artery in the fetal side of the umbilical cord (Fig. 3b). However, no TH-positive fibers were noted in the umbilical vein or Wharton jelly. NPY-positive fibers were noted also on the margins of the Wharton jelly (Fig. 2c).

In the fetal side of the umbilical cord, CGRP-positive fibers were distributed primarily on the outer surface of the smooth muscle layer of the umbilical artery (Fig. 4a). CGRP-positive fibers were detected also on the margin of the Wharton jelly (Fig. 4b). However, no CGRP-positive fibers were observed in the umbilical vein or Wharton jelly in the middle part or placental side of the umbilical cord.

In the fetal side of the umbilical cord, CGRP-positive fibers were distributed primarily on the outer surface of the smooth muscle layer of the umbilical artery (Fig. 4a). CGRP-positive fibers were detected also on the margin of the Wharton jelly (Fig. 4b). However, no CGRP-positive fibers were observed in the umbilical vein or Wharton jelly in the middle part or placental side of the umbilical cord. CGRP-positive fibers run in the Wharton jelly or from the umbilical artery to the outer surface of the smooth muscle layer (Fig. 4c). Table 2 summarizes the results of the Immunohistochemistry experiments.

DISCUSSION

The distribution of autonomic nerve fibers, and their relationship to vascular smooth muscle, in the human umbilical cord have not been elucidated.

The first study of peripheral nerves in the human umbilical cord was by Mabuchi in 1924 [3], followed by studies using silver impregnation by Konno [4] and Fujiyama et al. [5]. Studies using methylene blue vital stain by Fox and Jacobson [7] and by Jacobson and Chapler [8] have reported the presence of nerve fibers on the fetal side of the umbilical cord. Recently, the presence of nerves has been demonstrated in the human umbilical cord by various new techniques including a histochemical method using acetylcholinesterase (Ach), fluorescence microscopy, and electron microscopy. Among these reports, Matsubara and Tamada [1] demonstrated the presence of parasympathetic nerves by detecting Ach-positive fibers by electron microscopy, and Kawano and Mori [2] observed catecholamine-containing fibers in the fetal end of the umbilical cord by immunofluorescent staining. These catecholamine-containing fibers were distributed in the Wharton jelly near and between arteries and formed perivascular plexuses around the arteries on both sides. Kawano and Mori [2] also noted the presence of nerve fibers in the connective tissue surrounding arterial smooth muscle by electron microscopy and confirmed that they were sympathetic fibers by demonstrating the presence in them of granule-containing small vesicles, suggesting neural regulation of blood flow in the umbilical artery.

Recently, autonomic nerves containing the vasodilator peptide CGRP [9-11] and the vasoconstrictor peptide NPY [12-16] have been shown to be involved in vascular innervation. However, to our knowledge, there have been no reports demonstrating the presence of these vasoactive peptides in the human umbilical cord with the ABC method or fluorescence antibody method. In this study, we examined various parts of the human umbilical cord (fetal side, middle and placental side) immunohistochemically for CGRP-positive and NPY-positive nerve fibers. CGRP-positive and NPY-positive fibers were noted in the smooth muscle of the media of the umbilical artery and on the margins of the Wharton jelly in the fetal side of the umbilical cord. CGRP-positive fibers were distributed in a bead-like pattern and partly in a reticular pattern.

NPY-positive fibers and TH-positive fibers also showed a bead-like distribution. The area of their
Fig. 2. Photographs showing NPY-positive fibers. a, b, NPY-positive fibers (arrows) in the smooth muscle layer of the umbilical artery. c, NPY-positive fibers (arrows) in the Wharton jelly. UA: umbilical artery; WJ: Wharton jelly

Fig. 3. Photographs showing TH-positive fibers. a, b, TH-positive fibers (arrows) in the smooth muscle layer of the umbilical artery. UA: umbilical artery

Fig. 4. Photographs showing CGRP-positive fibers. a, CGRP-positive fibers (arrows) in the smooth muscle layer of the umbilical artery. b, c, CGRP-positive fibers (arrows) in the Wharton jelly. UA: umbilical artery; WJ: Wharton jelly
IMMUNOREACTIVE NERVE FIBERS distribution was confined within 10 cm from the umbilical ring, being consistent with Kawano and Mori report [6]. The number and thickness of these fibers decreased distally from the umbilical ring.

According to some investigators [1, 2], nerve fibers are present only in the umbilical artery and Wharton jelly but not around the umbilical vein or in the middle part or placental side of the umbilical cord. In the present study, also, CGRP-positive and NPY-positive fibers were not observed around the umbilical vein or in the middle part or placental side of the umbilical cord.

The tone of the resistance vessels, which plays an important role in the regulation and maintenance of the systemic arterial blood pressure, is considered to be maintained and regulated primarily by sympathetic nerves, which are vasoconstrictor nerves. Sympathetic nerves maintain and regulate the tone of the vessels via NPY contained in sympathetic nerve terminals as well as the neurotransmitter noradrenaline (NA) [13].

In this study, nerve fibers that contained TH, which is an essential enzyme of the noradrenaline synthesis pathway, were also examined by the ABC method and fluorescence antibody method. The distribution of these fibers was similar to that reported by Kawano and Mori [2].

Recently, blood vessels have been shown to be innervated by non-adrenergic, non-cholinergic vasodilators different from sympathetic or parasympathetic nerves. CGRP with a potent vasorelaxant action is involved in the regulation of the vascular tone as a neurotransmitter of vasodilator nerves [11].

In this study, we demonstrated the presence of CGRP-, NPY-, and TH-positive fibers around the umbilical artery in the human umbilical cord (on the fetal side). This finding suggests neural regulation of blood flow in the umbilical artery.

ACKNOWLEDGMENTS: The author would like to thank Prof. Michiaki Yakushiji and Dr. Shigeaki Iwanaga for their critical advice and encouragement during the course of this study.

REFERENCES


