Telocytes in Parotid Glands

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ABSTRACT
The parotid histological structure includes acinar, ductal, and myoepithelial cells, surrounded by a connective stromal component. The parotid stroma is mostly regarded as an inert shell, consisting of septa, which divide the parenchyma. Telocytes were recently identified as a new stromal cell type in various organs, including exocrine pancreas. We aimed to evaluate telocytes presence in parotid stroma and whether their topographical features might support an involvement in parotid function modulation. Serial ultrathin sections of human and rat parotid glands were studied and compared by transmission electron microscopy. Two-dimensional concatenation of sequenced micrographs allowed the ultrastructural identification of parotid telocytes, with their specific long, thin, and moniliform prolongations (telopodes). Telocyte location appeared frequently as a strategic one, in close contact or vicinity of both secretory (acini and ducts) and regulatory (nerves and blood vessels) apparatuses. They were also found in the interacinar and the subductal stroma. Two previously reported telocyte markers (c-kit/CD117 and vimentin) were assayed by immunohistochemistry. Actin expression was also evaluated. Telocytes are making a network, especially by branching of their long telopodes. Elements of this telocyte network are interacting with each other (homocellular connections) as well as with other cell types (heterocellular connections). These interactions are achieved either by direct contact (stromal synapse), or mediated via shed microvesicles/exosomes. Since telocyte connections include both neurovascular and exocrine elements (e.g., acini and ducts), it is attractive to think that telocytes might mediate and integrate neural and/or vascular input with parotid function.

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The parotid glands (PGs) produce up to 90% of human saliva (Garant, 2003) and are the largest of the three main salivary gland (SG) pairs. The shortest description for SG is that of several secretory units connected to the oral cavity by a system of ducts. On one hand, we know that salivary secretory units are organized in acinar (round) or tubular (elongated) cluster of cells (Tandler, 1993). On the other hand, we also know that terminal salivary secretory units merge to form small intercalated ducts, which drain into larger striated ducts, and finally merge into interlobular ducts. Secretory elements, together with their associated ductal segments, are organized into lobules (Riva et al., 1993) and therefore PG appears as an assemblage of lobules, surrounded by a plain stroma.

Stromal compartment of the PG is still not well known. A novel stromal cell type was recently described (Popescu and Faussone-Pellegrini, 2010)—the telocytes (TCs)—possessing the ultrastructural features for intercellular signaling (Popescu and Faussone-Pellegrini, 2010; Faussone-Pellegrini and Popescu, 2011; Popescu, 2011a). The TC presence was reported in the stroma of several organs, for example, heart (Bani et al., 2010; Faussone-Pellegrini and Bani, 2010; Gherghiceanu et al., 2010; Gherghiceanu and Popescu, 2010; Kostin, 2010; Popescu et al., 2010; Suciu et al., 2010a; Zhou et al., 2010), pla-centa (Suciu et al., 2010b), duodenum (Cantarero Carmona et al., 2011), lungs (Popescu et al., 2011a; Zheng et al., 2011b), pleura (Hinescu et al., 2011), pancreas (Nicolescu et al., 2010; Nicolescu and Popescu, 2011), and skeletal muscle (Bojin et al., 2011; Popescu et al., 2011b).

TCs have remarkably long, thin, and moniliform processes, called telopodes (Tp) (Popescu and Faussone-Pellegrini, 2010). The identification of TC is mostly based upon recognition of their Tp, which have a series of distinctive characteristics: length, thinness, moniliform aspect and a branching network pattern, forming a labyrinthine system by 3D convolution and overlapping, communicating through junctions (Popescu and Faussone-Pellegrini, 2010; Faussone-Pellegrini and Popescu, 2011; Popescu, 2011a). To observe a Tp, one should consider its main ultrastructural features. First of all, Tp tortuous trajectory requires convenient section planes, in which a larger/longer portion of a Tp to be enclosed. Secondly, since Tp are both very long and very thin structures, a both large and detailed image is required. In other words, a higher magnification overview is needed. Several neighbor areas should be analyzed under electron microscope and the captured images aligned and merged both horizontally and vertically. The reconstructed collage will show an area that could not have been directly captured entirely unless at lower magnification. In such way, a wider “field of view” may be analyzed at a higher magnification.

The moniliform aspect is ultrastructurally given by the alternation of thin fibrilar-like segments (podomers) and dilated, cisternae-like regions (podoms), rich in caveolae, endoplasmic reticulum, and mitochondria (Suciu et al., 2010a; Popescu, 2011b). The mean width of podomers is around 60 nm (Nicolescu and Popescu, 2011), below the resolving power of light microscopy. That is why electron microscopy analysis is mandatory for positive TC identification and complete ultrastructural characterization.

Immunohistochemistry (IHC) showed that TC were positive for c-kit/CD 117 and vimentin (Popescu and Faussone-Pellegrini, 2010; Suciu et al., 2010b). Another distinctive TC feature might be considered the shedding of microvesicles/exosomes, likely carriers of small RNAs, including regulatory micro-RNAs (Cismasiu et al., 2011; Record et al., 2011).

Here, we report (presumably for the first time) that TCs are present in the PG stroma. We suggest that their topography would allow them to participate in process of heterocellular signal transmission.

**MATERIALS AND METHODS**

**Human PG Samples**

Small fragments of human parotid glandular tissue, remote of primary disease, were collected at time of surgery from three patients with non-neoplastic disease. A group of samples (about 1 mm⁢³ each) were processed for transmission electron microscopy (TEM) according to routine procedures, as we previously described (Mandache et al., 2007; Gherghiceanu and Popescu, 2010; Popescu et al., 2010; Suciu et al., 2010b).

The other samples were fixed in 10% formalin until processed for IHC. Two archived paraffin blocks with embedded tumor-free human parotid glandular tissue were also used for IHC analysis. All patients provided informed written consent and the Institutional Ethics Committee approved the study.

**Rat PG Samples**

Under sodium pentobarbital anesthesia (60 mg/kg), rats were perfused with PBS at 50 ml/min for 2 min at room temperature and then with a freshly prepared solution of 2.5% glutaraldehyde in 15 mM cacodylate buffer, pH 7.2, for 5 min. The PGs were quickly excised and immersed 90 min, at 4°C, in the same fixative.

**Transmission Electron Microscopy**

Parotid tissue samples (fragments of about 1 mm³) were fixed with 1% osmium tetroxide in 15 mM cacodylate buffer, pH 7.4, for 90 min at 4°C, and dehydrated in a series of graded ethanol. After immersion in propylene oxide (three times for 10-min each), the samples were immersed in a mixture (1:1) of propylene oxide and Epon 812 resin, overnight, and embedded in Epon 812 as usually. The regions to be studied were sectioned using a MT-7000 ultramicrotome (from Research Manufacturing Company, Tucson, AZ) into ultrathin sections (50 nm). The sections were mounted on Formvar coated copper slot grids, stained with uranyl acetate and Reynolds lead citrate. They were examined using a CM 12 Philips electron microscope at an acceleration voltage of 60 kV. Digital electron micrographs were recorded with a Morada 11 Megapixels CCD using iTEM-SIS software (Olympus, Soft Imaging System GmbH, Germany).

**Two-Dimensional Reconstructions**

Images of several neighbor areas were captured at high magnification, then aligned both horizontally and vertically, and merged in a bidimensional reconstruction.
To increase the visual contrast between several structures on the same electron micrograph, we have digitally colored specific elements (e.g., TCs, telopodes, and nerves) to make them more visible for the untrained eye. All the elements were carefully hand colored in Adobe Photoshop software (Adobe Systems) using a Wacom digital tablet (Wacom Europe GmbH, Krefeld, Germany) to avoid any software mismatches.

**Immunohistochemistry**

IHC paraffin sections were deparaffinized, rehydrated, and rinsed in PBS pH 7.4. For CD117, microwave antigen retrieval was performed in TRIS/EDTA buffer, pH 9, for 5 min at 800 W and for 10 min at 440 W. The human parotid selected samples were tested by IHC using the following antibodies: polyclonal CD117 (1:100, DAKO, Glostrup, Denmark) and monoclonal smooth muscle α-actin/SMA (1:1,500, clone 1A4, Sigma Chemica, St. Louis, MO) and vimentin (1:100, clone V9, Dako, Glostrup, Denmark). All specimens were counterstained with Meyer’s hematoxylin, examined, and photographed on a ZEISS working station including an AxioImager M1 microscope with an AxioCam HRc camera, and the AxioVision digital image processing software. IHC was performed on 3-µm thick sections from 10% formalin fixed paraffin-embedded (arrows) often have a multipolar appearance sending prolongations in the periphery of the neighboring acini. Right panel: Higher magnification gives a more clear view on cytoplasmic processes of vimentin positive cells indicated by arrows.

**RESULTS**

**Immunostaining**

Vimentin. Immunostaining for vimentin identified positive stromal cells. Their morphology varied from spindle-shaped/bipolar to multipolar, in many instances with long prolongations with a moniliform appearance. We distinguished two vimentin-positive cells localizations: septal and periacinar (Fig. 1). The first group contributed to extensive septal networks around ducts and blood vessels. Vimentin-positive periacinar cells were also participating in stromal networks by circumventing PG acini.

**c-kit/CD 117.** Solitary c-kit/CD117 positive multipolar interstitial cells were identified within the intraparotid
large septa, in periductal and perivascular locations (Fig. 2). As their processes were accurately detected, we considered these cells as being c-kit/CD117 positive stromal cells, and not mast cells, which do not usually present any prolongations. Some prolongations also appeared moniliiform, as Tp. c-kit/CD117 immune labeling did not identify any positive element within the parotid acini or neighbouring the ducts smaller than the interlobular ones. Some nonspecific binding in Fig. 2 occurred in ductal epithelial cells; however, our focus was on interstitial positive elements.

Smooth muscle α-actin. Immune labeling for SMA identified myoepithelial cells, disposed on the basal laminae of the secretory acini (arrowheads in Fig. 3).

Long prolongations (tens of micrometers) of SMA-positive cells seemed to form periductal continuous networks. These serial-linked Tp (arrows in Fig. 3) had a total length in the range of hundreds of micrometers. Moreover, similar positive cells were located between the acini, outside their basal laminae, sending long prolongations between and around PG acini.

Electron Microscopy

Electron microscopy confirmed the presence of cells with ultrastructural features of TC in locations similar to IHC results. The morphological and ultrastructural pattern of the parotid TC consisted of:

1. presence of caveolae (Figs. 4,5),
2. an euchromatic nucleus (Figs. 4–6),
3. interrupted appearance of the basal lamina, and
4. presence of Tp, with a regular moniliform aspect, alternating thin segments (podomeres) with dilations (podoms)—rich in mitochondria and endoplasmic reticulum (Figs. 4,5).

Within the interlobular septa, solitary spindle-shaped TC were identified in both rat and human samples (Fig. 4), sending long Tp, with caveolae and an uneven caliber (Figs. 4,5). TCs were mostly found in the interlobular septa (Fig. 4) and between the PG acini (Fig. 5), in accordance with immune positive cells loci identified by IHC.

Mast and plasma cells were also spotted on TEM micrographs. The stromal synapse between a long Tp and a mast cells is shown in Fig. 6 (and in Supporting Information 3). In an inset of Fig. 4, we show a stromal synaptic line more than 3-µm long between a TC and an acinar cell. Evidence of shed microvesicles/exosomes was noted at a slighter lower rate compared with pancreatic tissue (Nicolescu and Popescu, 2011).

Complete loops of Tp were surrounding intraparotid nerves of various calibers (Fig. 7 and Supporting Information 1), mostly amyelinic. These stromal TC-nerve
complexes neighbored, variably, secretory elements—ducts and/or glandular acini (Fig. 7) and various caliber blood vessels, from capillaries to arteriolae (Fig. 8 and Supporting Information 2).

Extremely long periacinar Tp were outside to the acinar basal lamina, while myoepithelial cells were identified inside to the lamina (Fig. 9), an important topographical distinction. Collagen fibrils bundles at various section angles are surrounded/systematized by multiple long parallel Tp and why they were overlooked so far. Asterisks indicate TC caveolae. Ac, acinar cell; TC, telocyte; Tp, telopode.

**DISCUSSION**

Between the two parotid lobes (deep and superficial) lies the cranial nerve VII, branching its motor tributaries inside the PG. The degree in which this nerve adheres to PG tissue is of outmost importance during surgery and critical for postoperatively quality of life. Thus, it is very important to elucidate the ultrastructural aspects of internal PG organization, especially for perineural cellular elements. It was proven that PG stroma forms a microenvironment that is fundamental for the homeostasis of this organ and that PG homeostatic mechanism is better than in the submandibular gland (Ferragut et al., 2011).

The PG and the exocrine pancreas have similar structural properties (Take et al., 2007). We have already found stromal TCs in the pancreatic interstitium (Nicolescu et al., 2010; Nicolescu and Popescu, 2011), so looking for them in PG tissue was our next approach. But instead of founding them only in periacinar localization, we encountered parotid TC also bordering various ductal segments of various calibers, from striated to interlobular ducts. We aimed to superpose the topographical pattern of TC over the normal map of stromal constituents. This would allow the presumption of a possible TC modulation of the parotid function. Ducts-related interstitial cells were also reported at the level of the extra hepatic (Hinescu et al., 2007; Ahmadi et al., 2010) and intrahepatic bile ducts (Rusu et al., 2011), and of the urogenital tract (Lang et al., 2006; Metzger et al., 2008; Hashitani and Lang, 2010).

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2Stromal synapses represent probably the main TC heterocellular communication mechanism. See Popescu et al., 2005, for reference.
The vimentin positive phenotype supports the mesenchymal origin of parotid TC. Their long processes, Tp, are rich in actin filaments. Light microscopy requires a c-kit/CD117 positive expression of cells with at least two processes. These prolongations exclude mast cells. We found both unipolar and multipolar positive TC.

The ductal system also contains the tissue stem/progenitor cells (Denny and Denny, 1999; Man et al., 2001; Lombaert et al., 2008a). A population of CD117/c-kit-expressing cells was found in mice, with remarkable capability to restore SGs damaged by radiation (Lombaert et al., 2008a). It was also shown that, in vivo, expansion of SG stem cells is attempted (Lombaert et al., 2008b), and it may thus also be possible in vitro. It was also demonstrated that human SGs contain a similar “putative” stem cell population as rodents, expressing CD117/c-kit and capable of in vitro differentiation and self-renewal (Feng et al., 2009). Since our interest was focused on CD117/c-kit positive elements in the interstitium, the immune labeling of ductal epithelium was not specifically addressed to.

It was found that in rats none of the salivary ducts—parotid, mandibular, or sublingual—are innervated directly (Templeton and Thomas, 1982). However, there are four categories of nerve function in relation to SGs, including water mobilization, protein secretion, induction of synthetic activities, and maintenance of mature cells in a functioning size and state (Garrett and Kidd, 1993). Thus, an interstitial pathway of signaling, as suggested by our results, can...
be assumed. In ferrets, nerve trunks were identified within the interlobular stroma of the submandibular gland.

Additionally, an extensive intralobular nerve fibers network was evidenced embracing acini and ducts. Stellate cells with fine branching processes embracing acini were presumed as being myoepithelial cells, and a few spindle-shaped basal cells, investing striated ducts, were demonstrated and were considered to confer mechanical stability and/or contractile ability (Fletcher et al., 1999). Myoepithelial cells are usually located around intercalated ducts (Ogawa, 2003) and extend their processes around the acini, but they have also been found in acini (Garrett and Parsons, 1973) or striated ducts (Garrett, 1967; Cutler et al., 1977). Since some myoepithelial cells have been reported in the vicinity of the acini (Chaudhry et al., 1987), a differential diagnosis with TCs should be taken into account. In this study, although myoepithelial cells were positive for actin too, the differential diagnosis for periacinar cells with prolongations considered their typical localization as referred to the acinar basal lamina (Fig. 2, IHC and Fig. 9, TEM).

It was being hypothesized that striated ducts may be involved in other roles beside electrolyte homeostasis (Tandler et al., 2001). Small microvesicles/exosomes shed by TC could also influence the striated ducts activity, as well; especially, since vesicular communication plays an important role there (Tandler and Phillips, 2000).

However, for a positive TC diagnosis, TEM investigation is mandatory. The unique moniliform Tp appearance might be explained by the particular podoms structure, which offer energetic (mitochondria), structural (proteins), and functional (caveolae) support for the entire Tp.

The molecular mechanism that underlies the conspicuous morphological aspect of parotid TC is yet to be revealed. The difference noted in the number of shed microvesicles/exosomes found in TC/Tp areas compared with pancreatic interstitium might be explained by the different topography of TC/Tp locations. While pancreatic TC were mainly present around and between acinar units, in PG we encountered them also around ducts of various calibers. Taking into account that shed microvesicles/exosomes are involved in intercellular signaling (Record et al., 2011), we might presume that parotid TC (especially periductal ones) exchange information mainly by direct contact/stromal synapses, ergo the reduced amount of microvesicles. The purpose of this study was mainly to present (presumably for the first time) the morphological and topographical features of TC in PGs. Possible implications of TC in pathology were recently addressed (Mandache et al., 2010; Zheng et al., 2011a). We hypothesize that, since TCs interact with almost all parotid structures, they may be the stromal elements that contribute in maintaining “PG homeostasis.” Further investigation of the signaling networks of TCs and their relation(s) with neighboring cells might be helpful in both physiological and pathological states.

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LITERATURE CITED


