Telocytes in trachea and lungs

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Abstract

We show the existence of a novel type of interstitial cell—telocytes (TC) in mouse trachea and lungs. We used cell cultures, vital stainings, as well as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and immunohistochemistry (IHC). Phase contrast microscopy on cultured cells showed cells with unequivocally characteristic morphology of typical TC (cells with telopodes—Tp). SEM revealed typical TC with two to three Tp—very long and branched cell prolongations. Tp consist of an alternation of thin segments (podomers) and thick segments (podoms). The latter accommodate mitochondria (as shown by Janus Green and MitoTracker), rough endoplasmic reticulum and caveolae. TEM showed characteristic podomers and podoms as well as close relationships with nerve endings and blood capillaries. IHC revealed positive expression of TC for c-kit, vimentin and CD34. In conclusion, this study shows the presence in trachea and lungs of a peculiar type of cells, which fulfils the criteria for TC.

Key words: telocytes ● telopodes ● interstitial cells ● stromal cells ● lungs ● trachea ● c-kit ● CD34 ● vimentin

Introduction

Telocytes (TC) are a novel type of interstitial cells firstly described in 2005 by Popescu *et al.* [1]. Initially, they were named interstitial Cajal-like cells (ICLC), but when the lack of similarity with canonical interstitial cells of Cajal (ICC) became obvious, the name *Telocytes* was introduced [1, 2, 18], and is currently used [2–25]. The presence of TC was documented in various cavitary organs (heart—endo- [7], myo- [26–29], pericardium [30, 31], pulmonary veins [32], intestine [17], mesentery [33], gall bladder [34], uterus and fallopian tube [35–37]) and non-cavitary organs (lungs [24], pleura [22], skeletal muscle [19], exocrine pancreas [38], mammary gland [39–41] and placenta [8,42]. The general aspect of TC is of a cell with small cell body and long processes called telopodes (Tp). Therefore, the shortest definition for TC is: cell with Tp.

The aim of this work is to prove the existence of a new type of cells, TC in stroma of trachea and lungs. We confirm the existence of TC in lungs, as previously reported by Popescu *et al.* [24]. Using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and cell cultures [phase contrast microscopy and immnunohistochemistry (IHC)] we found characteristic TC (Tp, podomers, podoms).

Material and methods

Animals

We used BABL/c mice, taken from the animal research centre of Fudan University (Shanghai, China). Mice weighed 20–25 g (4–6 weeks). All mice were housed in a local facility for laboratory animal care and held, fed *ad libitum* on stock diet, according to the local ethical guidelines. The study was approved by the Ethic Committee for Animal Care and Use, Fudan University, and according to generally accepted international standards.

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Isolation of TC from lung and primary cell culture

After BABL/c mice were killed with an overdose of anaesthetic. The trachea and lung fragments were isolated under sterile conditions and collected into sterile tubes containing DMEM (Gibco, NY, USA), supplemented with 100 UI/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich Shanghai Trading Co Ltd., Shanghai, China), placed on ice and transported to the cell culture laboratory. Samples were processed within 30 min. from surgery, rinsing them with sterile DMEM. The lung was minced into fragments of about 1 mm^3 and incubated on an orbital shaker for 4 hrs, at 37°C, with 10 mg/ml collagenase type II (Sigma-Aldrich, St. Louis, MO, USA) and 2000 U/ml deoxyribonuclease I (Sigma-Aldrich) in PBS, without Ca^{2+} and Mg^{2+}. Dispersed cells were separated from non-digested tissue by filtration through a 40-μm-diameter cell strainer (BD Falcon, NJ, USA), collected by centrifugation at 2000 r.p.m., 5 min., and resuspended in DMEM, supplemented with 10% foetal calf serum, 100 UI/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich). Cell density was counted in a haemocytometer and viability was assessed using the Trypan blue dye exclusion test. Cells were distributed in 25 cm^2 plastic culture flasks, at a density of 1×10^5 cells/cm^2, and maintained at 37°C, in a humidified atmosphere (5% CO_2 in air) until becoming semi-confluent (usually 4 days after plating). Culture medium was changed every 48 hrs. Cell cultures were examined by phase contrast microscope, under an inverted Olympus phase contrast microscope (1X51; Tokyo, Japan), and the typical TC were photographed.

Stainings in cell culture

Methylene blue vital staining

Primary cultured TC in 25 cm^2 Corning culture flask were washed in pre-warmed phenol red-free DMEM, and incubated for 20 min., in a 0.02% methylene blue solution (Sigma-Aldrich), at 37°C, in a humidified atmosphere, 5% CO_2 in air, then TC were examined and photographed.

Giemsa staining

The 25 cm^2 Corning culture flask was emptied of the culture medium, and TC were stained with 0.4% Giemsa solution (Sigma-Aldrich) in methanol and distilled water (pH 6.9), and incubated for 30 min., at room temperature, and washed three times in distilled water, then TC were examined and photographed.

Janus green B vital staining

TC in 25 cm^2 Corning culture flask were stained for 30 min. in 0.02% Janus Green B (Sigma-Aldrich) in DMEM and maintained at 37°C, in a humidified atmosphere, 5% CO_2 in air. After repeated washes in DMEM, then TC were examined and photographed.

Mito tracker green FM was used for mitochondrial labelling

TC in 25 cm^2 Corning culture flask were incubated in phenol red-free DMEM completed with 10% foetal calf serum and labelled with 80 nM Mito Tracker Green FM (Beyotime C1048-50μg; Nantong, China), and incubated for 30 min., at 37°C, in a humidified atmosphere, 5% CO_2 in air, subsequently washed with DMEM medium, and examined and photographed by fluorescence microscopy (450–490 nm excitation light, 520 nm barrier filter; Olympus 1×51).

Scanning electronic microscopy

TC in trachea tissues

The trachea samples (after removing the epithelium) were fixed as follows: (1) 3% buffered glutaraldehyde for 3 hrs; (2) washing three times in PBS (10–15 min. each change) to remove all traces of glutaraldehyde; (3) post-fixation in 2% osmium tetroxide for 2 hrs; (4) dehydration through graded series of ethanols: 50%, 70%, 80%, 95%, 30 min. each change, 100% twice, 30 min. each change); (5) transfer to critical point dryer. TC were observed and photographed with a JEOL JSW-6390LV (Tokyo, Japan) scanning electron microscope, at 5 kV.

Transmission electron microscopy

The trachea was cut into small pieces about 1 mm^3, and they were fixed in 4% glutaraldehyde (pH 7.3, 4°C) for 4 hrs-washed in 0.1M cacodylate buffer-post-fixed with 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.3, 4°C)—dehydrated in a graded series of ethanols—impregnated in propylene oxide (immersed overnight in a mixture of propylene oxide and Epon 812 resin) and embedded in Epon 812. Ultrathin sections 70 nm were cut on a Leica LKB-II (Nußloch, Germany). Sections were collected on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and observed at an acceleration voltage of 80 kV, in JEOL JEM-1230 (Tokyo, Japan) electron microscope.

Immunohistochemistry (IHC)

Specimens of trachea and lungs were isolated and fixed with 10% neutral formalin and embedded in paraffin. The Abcam primary antibodies (Abcam, San Francisco, CA, USA) were used as follows: c-kit, rabbit polyclonal, 1:100; vimentin, rabbit polyclonal, 1:100; CD34, rat monoclonal, 1:100. Negative controls were performed by omitting the primary antibody or by substitution of the primary antibodies with non-immune serum in an otherwise similar protocol. Tissue sections were examined and photographed under an Olympus light microscope (BX51) equipped with a digital camera (Olympus dp71; Olympus, Tokyo, Japan).

Results

Identification of TC in the trachea and lungs were based on a set of complementary morphological approaches. Each method was exploited to provide data for either the positive or differential diagnosis of TC, and the most of characteristic features were examined comparatively.

## Primary cell culture and vital stainings

TC were successfully maintained in primary culture and could easily be identified before reaching confluence. Starting from the third to fourth day in culture, TC appears with typical Tp: long, and with uneven caliber: an alternation of podoms and podomers (Fig. 1).
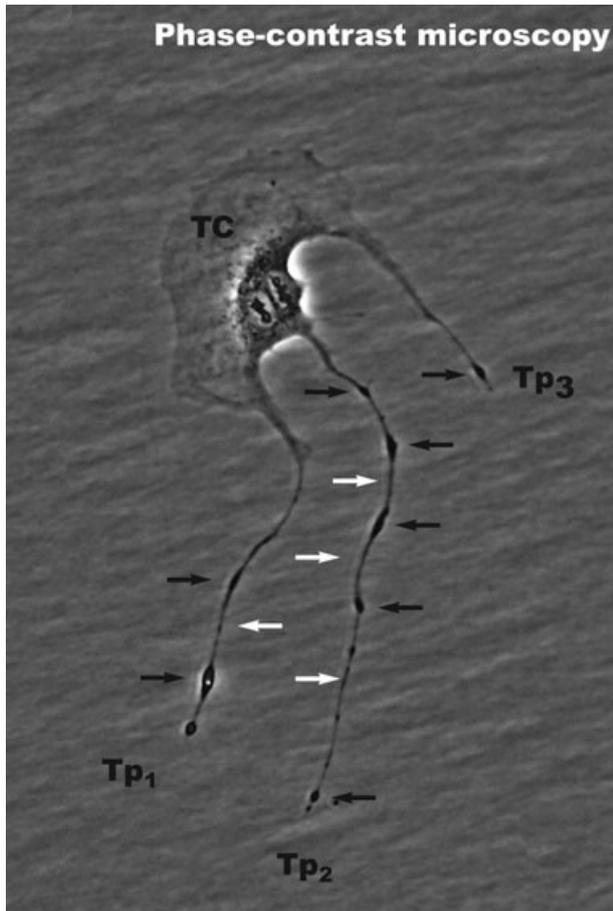
Fig. 1 Phase-contrast microscopy; TC in primary culture; mouse lung. A typical telocyte is depicted with three long and slender Tp. Tp characteristically present the alternation of thick segments—podoms (black arrows)—and thin segments-podomers (white arrows).
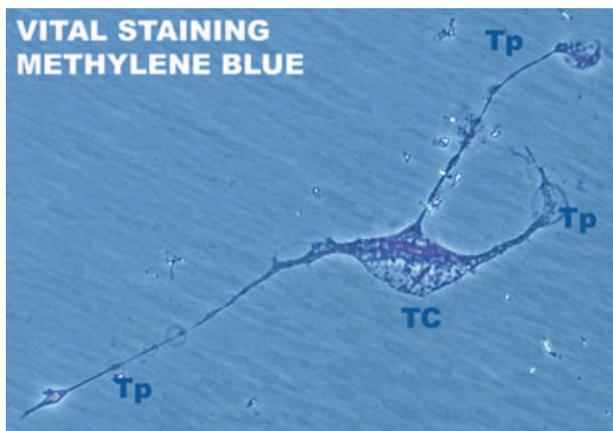
Fig. 2 Phase-contrast microscopy; living TC in primary culture; vital staining with methylene blue; mouse lung. A typical TC with a pyramidal cell body and three long Tp. The alternation of podoms and podomers is characteristic for the Tp. Original magnification: 200×
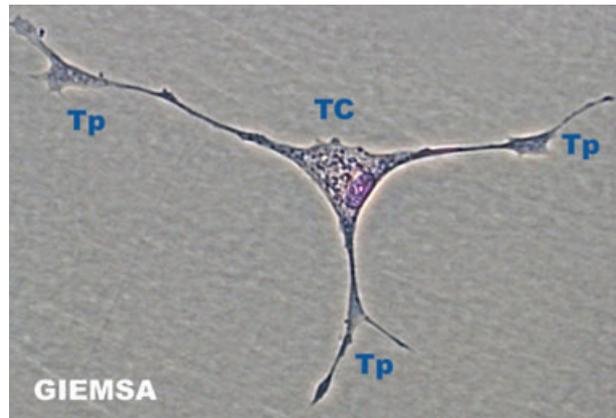

Fig. 3 Mouse lung; TC in culture; Giemsa staining. A typical TC with a pyramidal cell body and with three long Tp. The alternation of podoms and podomers is striking for the Tp. Dichotomic branching of Tp is obvious. Original magnification: 200×
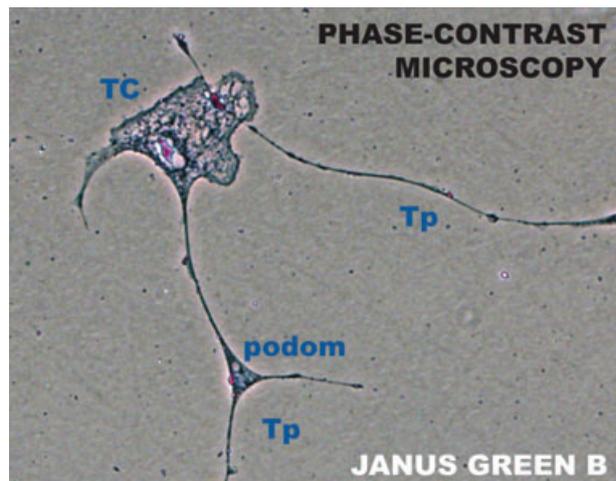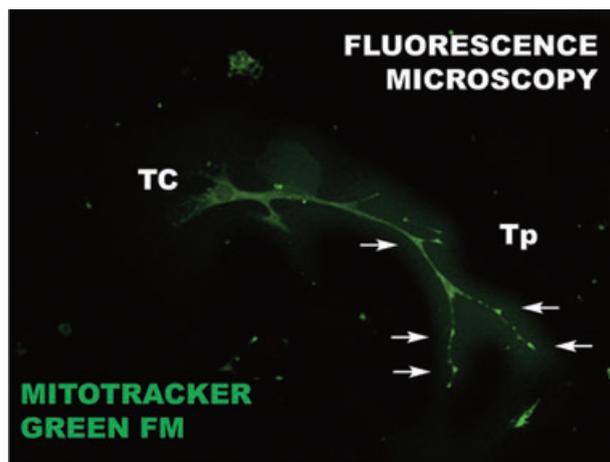

Fig. 4 Vital staining with Janus Green B; primary cell culture; mouse lung. A typical TC with two branched Tp. The vital staining shows accumulation of mitochondria within TC cell body and as well as the level of podoms. A long Tp, belonging to another TC is shown in the upper part of the figure, overpassing the cell body of the other TC.

Methylene blue vital staining and Giemsa staining revealed TC. (Figs 2 and 3) Tp are very long and have an uneven caliber, with dilated portions, resembling 'beads on a string'.

Janus green B is a classic vital staining with high affinity for mitochondria and it is used to assess viability and localize mitochondria. The initial dark green-blue colour, due to mitochondria stained with Janus green B, became a brownish-grey one. Figure 4 illustrates the TC body and Tp with podoms highly stained, indicating the presence of mitochondria in cell body, and particularly in podoms. *Mito Tracker Green FM* is a molecular probe with high affinity for michondrial membranes, and therefore
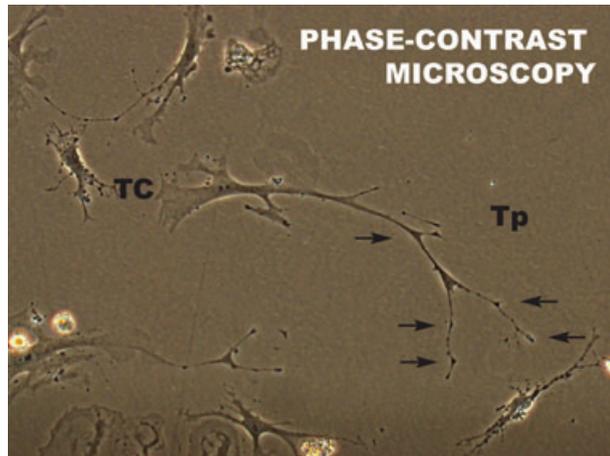
Fig. 5 Primary cell culture; mouse lung. Phase-contrast microscopy shows canonical TC with branched Tp. The uneven caliber (alternation of podoms—arrows—and podomers) of the Tp is observed. Original magnification: 100×

Fig. 7 Scanning electron microscopy of the lamina propria of mouse trachea. The tracheal epithelium was removed. The complex interstitial cell 'landscape' is obvious. Note a fibroblast with short prolongations and a dilated cell body. At least two silhouettes suggestive for highly branched TC are detectable. Scale bar: 10 μm.
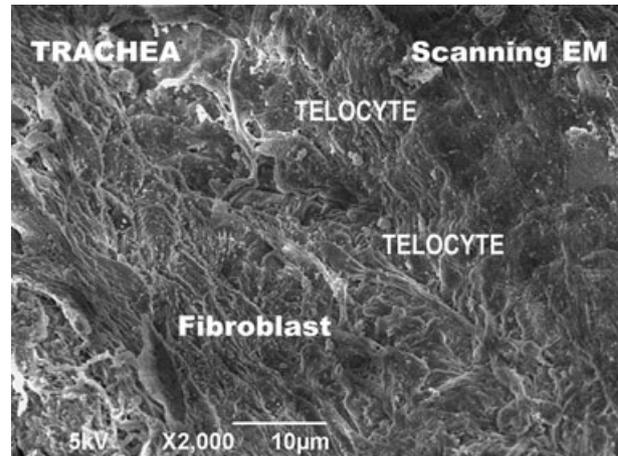

Fig. 6 Fluorescence microscopy; MitoTracker Green staining; mouse lung; primary cell culture. The mitochondria of the same living cell from Figure 5 are revealed and mainly within cell body and podoms (arrows). Original magnification: 100×

used to identify mitochondria in living cells. Compare Figures 5 and 6 and see that what appears as podoms under phase-contrast microscopy (Fig. 5) is revealed as an accumulation of mitochondria by using MitoTracker Green FM (Fig. 6).

Scanning electron microscopy

After the removal of tracheal epithelium, SEM reveals in lamina propria a complex cellular 'landscape' (Fig. 7). However, several cell types can be identified by their characteristic silhouettes. For instance, a fibroblast is recognized as well as at least two TC with abundant branched TP.
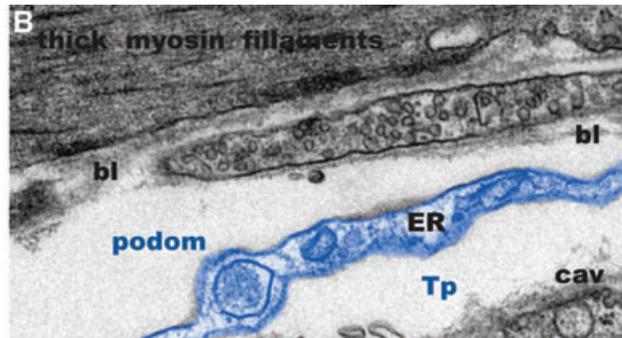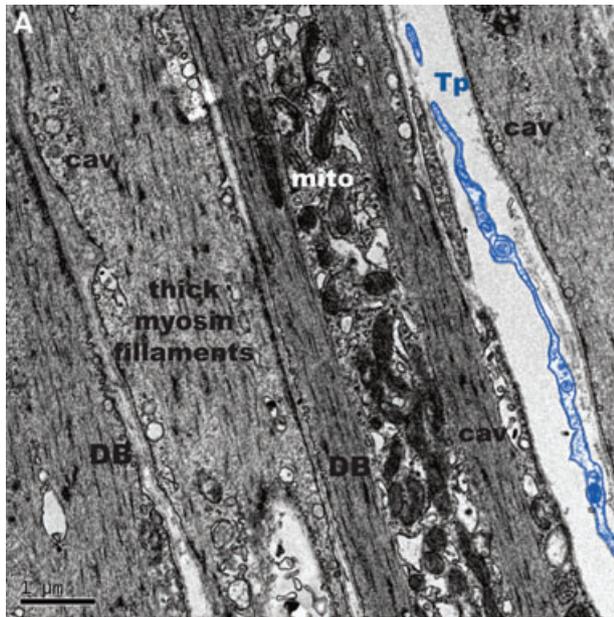
Transmission electronic microscopy

Transmission electronic microscopy revealed smooth muscle cells with dense bodies, thick myosin filaments, caveolae and numerous mitocondria at the pole of the nucleus (Fig. 8A). Two fragments (the upper—shorter, the lower being longer), most probably belonging to the same Tp are situated in the interstitial space, between two smooth muscle fibers. Figure 8B shows, at higher magnification, a detail of Figure 8A: a fragment from the endothelium (lined by basal lamina) is visible, having caveolae on both sides. Smooth muscle fibers (having thick myosin filaments) are also present and they have basement membrane. Among smooth muscle fibers and endothelium a Tp is present. Tp has a visible podom (containing one mitochondrion, and elements of endoplasmic reticulum—ER) as well as podomer.

Figure 9A shows different spatial relations of three distinct Tp with nerve endings within the interstitial space of an intralobular bronchiole. Typical Tp (with podoms) surround the nerve endings. Figure 9B also shows the close relationships of Tp with different cells.

Immnunohistochemistry

Double immunostaining for c-kit and vimentin (Fig. 10A) demonstrates the presence of a typical TC morphology (having very long and very thin Tp) running parallel in the vicinity of the lumen of a small vein. Figure 10B shows positive expression of TC for CD34. TC is located in the interstitial space between the airway epithelium and the endothelium of the blood vessel. CD34 is expressed in TC cell body and in cell prolongations.

Fig. 8 (**A**) Mouse trachea; transmission electron microscopy of smooth muscle layer. Note, four muscle cells longitudinally cut and in between two fragments (one long, one short) of a Tp. It is to be mentioned the presence of thick myosin filaments in smooth muscle cells. DB: dense bodies; mito: mitochondria. (**B**) Higher magnification of a fragment from (**A**). Note, a smooth muscle cell with thick myosin filaments and basal lamina (bl); a fragment of a telopode, a podom containing element of ER. cav: caveolae; bl: basal lamina.

Fig. 9 (**A**) Mouse lung; transmission electron microscopy; interstitial space of a intralobular bronchiole. Note the presence of Tp in close contacts with obliquely sectioned nerve endings. (**B**) Bonchoalveolar junction. Three fragments of a Tp are visible among local cellular population.
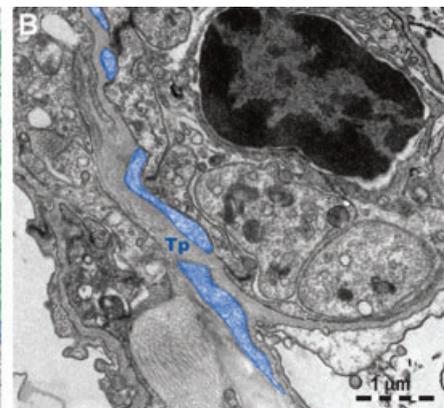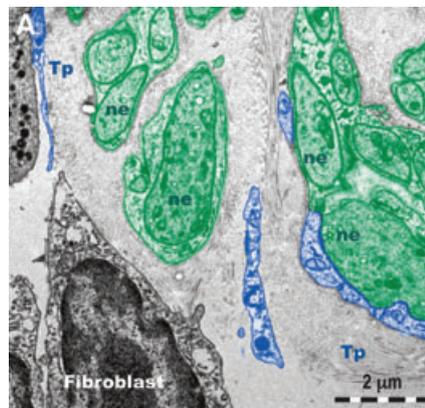
Fig. 10 (**A**) Immunohistochemical reaction for c-kit and vimentin in lung tissue. Long TC silhouette is seen positive near small vein lumen. (**B**) Positive reaction for CD34 of TC between airway epithelium and blood vessel endothelium.
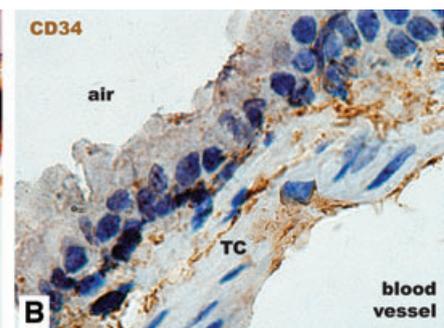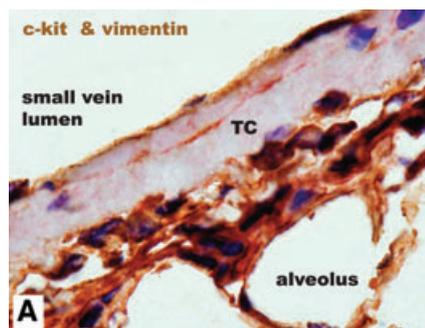
Discussion

In the last few years numerous data were reported showing the existence of a novel cell type namely *Telocytes* (TC) (previous synonym Interstitial Cajal-Like Cells, ICLC); for reviews see Refs. [1, 2, 18]. TC are interstitial (stromal) cells located in connective tissue, among other specific cellular types such as epithelial, muscular, or nervous cells. Recently, it was reported the finding of TC in pleura and lungs

[22, 24]. Therefore, our study confirms the existence of TC in lungs, and in addition demonstrates the presence of TC in trachea.

The results reported here show unequivocally that the cells we are describing in trachea and lungs fulfil the criteria for the positive diagnosis of TC, according to refs. [1, 2, 18]. These cells have a small cell body, and several (two to three) prolongations, namely Telopodes (Tp). These processes are very specific for TC.

Tp are characterized by the following features:

(1) Their very long, usually tens of micrometers, but frequently one hundred or more micrometres. However, cell culture and SEM have the advantage to show almost the entire TC, in comparison with sections for TEM, which contain only fragments of prolongations.

(2) They are made by an alternation of dilated portions, named *podoms* (containing mitochondria and endoplasmic reticulum) and *podomers* (thin segments). The thickness of podomers is usually very thin, below the resolving power of light microscopy in conventional sections.

(3) Dichotomous branching is extensive.

(4) Immunohistochemistry: positive reactions for c-kit, vimentin and CD34.

Our data do not provide direct information about the presumptive TC function(s). However, besides the conventional role of mechanical support for the TC network, we think that TC main role is intercellular communication and regulation. This is based on the existence of Tp 3D network. Noteworthy, the location of TC and Tp in close vicinity of blood capillaries and/or nerve endings support this assumption.

To understand TC role(s), further work is clearly required using dynamic methods.

Acknowledgements

Conflict of interest

The authors declare no conflict on interests.

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