

## Isolation and characterization of guinea-pig tracheal smooth muscle cells that retain differentiated function in long-term subculture \*

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**Summary.** A simple 30-min enzyme digestion procedure has been used to release guinea-pig tracheal smooth muscle cells that retain differentiated function in long-term subculture. Primary cell cultures initially consist of numerous epithelial colonies and 70–1000 morphologically differentiated smooth muscle cells per 600 mg (wet weight) tracheal tissue depending on the age of the animal. Both cell types proliferate to form a confluent monolayer within 5–17 days. Pure subcultures of tracheal smooth muscle cells are obtained by limited trypsin digestion of the primary culture. Eighty percent of these subcultured smooth muscle cells retain the ability to contract in response to histamine ( $10^{-6}$  M) and to form reaggregates even after 20 or more passages. Examination of these cells by electron microscopy reveals both biosynthetic and contractile components of smooth muscle. Analysis of this dual phenotype may provide valuable information about the regulation of tracheal smooth muscle cell growth and differentiation.

**Key words:** Trachea – Smooth muscle cells – Subculture – Response to histamine – Hartley guinea-pig

Viable smooth muscle cells (SMCs) of vascular and visceral origin have been released from tissue matrices of several mammalian species by various enzyme digestion techniques (Purves et al. 1973; Chamley and Campbell 1975; Chamley et al. 1977; Rifas et al. 1979; Avner et al. 1981; Ionasescu and Ionasescu 1985). Modulation of these differentiated SMCs from a quiescent contractile state to a synthetic state characterized by secretion of extracellular macromolecules and intense cell proliferation has been well documented (Fritz et al. 1970; Chamley-Campbell et al. 1979, 1981; Palmberg et al. 1985). Interestingly, several reports indicate that proliferating vascular SMCs from human, monkey, and rabbit sources do not contract in response to mechanical or pharmacologic stimuli (Martin and Sprague 1973; Campbell et al. 1974; Mauger et al. 1975; Chamley et al. 1977), nor can thick myosin filaments be demonstrated (Chamley and Campbell 1975). Restoration of contractile

competence has been achieved in primary cultures (Chamley-Campbell et al. 1979; Gunther et al. 1982) of SMCs by arresting cell proliferation. The inability to demonstrate a contractile response in proliferating cell cultures may reflect a transient loss of specific receptors and/or a disruption of the contractile apparatus. This lack of differentiated function has limited the usefulness of these cultured cells for studying biochemical mechanisms of smooth muscle-cell contraction. A uniform population of nontransformed SMCs capable of maintaining a contractile response in long-term subculture would therefore be a major advantage.

Recently, Avner et al. (1981) have reported the culture of canine tracheal SMCs in vitro. Thick myosin filaments were demonstrable only in primary SMC cultures where the plasmalemma had been disrupted by chemical manipulation. These tracheal SMCs were responsive to  $10^{-4}$  M carbachol stimulation between day 2 and day 8, with a diminished response after day 9 in primary culture. Neither SMCs in long-term primary culture nor subculture were tested for contractile response.

We report a simple, reproducible, 30-min enzymatic digestion that releases SMCs and epithelium from the guinea-pig trachea. In primary culture these SMCs exhibit a syncytial response to  $10^{-6}$  M histamine. Both proliferating (log phase) and nonproliferating (stationary phase) subcultures of these SMCs retain this ability to contract after 20 or more passages, indicating that both histamine receptors and the contractile apparatus are present and functional. Ultrastructural characteristics of both "contractile" and "synthetic" smooth muscle, including myofilaments, dense bodies, plasmalemmal vesicles, basal lamina, numerous polyosomes, mitochondria and rough endoplasmic reticulum, were all observed in these subcultured cells.

### Materials and methods

Adult male Hartley guinea-pigs (250–600 g) were sacrificed by pentobarbital sodium (500 mg/kg). Tracheae were excised aseptically and immediately rinsed twice with ice cold Hanks' balanced salt solution (HBSS) supplemented with penicillin (100 U/ml) plus streptomycin (100 µg/ml) (P/S). All extraneous tissue was carefully stripped from the trachea by blunt dissection with frequent rinsing of the tissue in HBSS to prevent dessication. Tracheae (600 mg/digest) were cut longitudinally through the rings opposite the strip of smooth muscle (trachealis) and were incubated with

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gentle agitation in 2.0 mls of 0.2% collagenase, 0.05% elastase IV (Sigma, St. Louis, MO) in HBSS at 37°C for 30 min. Smooth muscle cells were collected by pooling this enzyme digest with 2 HBSS washes (2 volumes each) of the undigested tracheal tissue. Additional SMCs could be liberated by incubating the remaining trachealis and/or intercartilage tissue in 0.2% collagenase for 1–3 h. All reagents used for the dispersion and culture of smooth muscle cells were purchased from Gibco Labs, Grand Islands, NY, unless stated otherwise.

Cells were centrifuged at room temperature at 1200 rpm in a clinical centrifuge for 6 min. The pellet, containing single cells and sheets of epithelium, was resuspended in 2 volumes of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and P/S. Epithelial sheets settled to the bottom of the tube within 10–15 min and were easily dispersed into colonies ranging from 10–200 cells by gentle aspiration. Single cells and epithelial colonies were inoculated into two 25 cm<sup>2</sup> culture flasks and incubated at 37°C, 5% CO<sub>2</sub>: 95% air. Twenty-four to 36 h after plating, the flasks were scored for phenotypically differentiated smooth muscle cells by phase contrast microscopy.

At confluence, smooth muscle cells were selectively harvested from the epithelial colonies by washing the cells twice with 2 ml of HBSS without calcium and magnesium, followed by 1 ml of versene (1:5000) and then a 60-sec incubation in 1 ml of 0.25% trypsin at 37°C. Smooth muscle cell number and viability were determined by use of a hemocytometer and the trypan blue exclusion method (Tolnai 1975). Cells were subcultured at  $1 \times 10^4$  or  $1 \times 10^5$  cells per 25 cm<sup>2</sup> flask and grown to confluence with medium replenishment every 2 to 3 days.

To confirm that primary cultures and subcultures were smooth muscle and not fibroblasts several parameters of smooth muscle structure and/or function were analyzed: histamine response and EM ultrastructure. Confluent and subconfluent cultures of the presumed SMCs as well as guinea-pig and human dermal fibroblasts (courtesy of Dr. J. Shapiro, St. Vincent Hosp.) were challenged for 20 min with  $10^{-6}$  M histamine (Sigma) in HBSS at 37°C to elicit a contractile response. This response was determined visually by phase-contrast optics. Cells were subsequently rinsed twice with 3 volumes of HBSS and 1 volume of growth medium to induce full relaxation to the resting state. Cells were photographed on Kodak Panatomic-X 35 mm film by use of Nikon Diaphot photomicroscope.

Ultrastructural features of both trachealis muscle tissue and subcultured tracheal SMCs (log phase) were examined by electron microscopy. Freshly excised trachealis muscle was minced into 1–2 mm segments in phosphate-buffered saline, pH 7.4 and fixed at 37°C for 20–30 min in 0.1 M phosphate buffer, 2% glutaraldehyde, pH 7.4. Cell cultures were washed twice with 5 ml HBSS and similarly fixed. After 30 min, SMCs were gently scraped off the substratum into the fixative. Tissue and cell suspensions were pelleted and then resuspended in fixative for 30 min at room temperature. After spinning, the pellets were resuspended in a small amount of 0.1 M phosphate buffer containing several drops of melted agar. Both suspensions were pelleted and placed in fixative for an additional 20 min. The samples were cut into 1 mm<sup>3</sup> blocks and post-fixed for 30 min in 1% OsO<sub>4</sub> in 0.1 M phosphate buffer, pH 7.4, at room tem-

perature. Blocks were stained for 9 min in 10% uranyl acetate in 50% ethanol and then embedded in Epon and sectioned at 600 Ångströms. Sections were stained for 7 min in lead citrate and examined in a Zeiss EM 9S2 electron microscope.

## Results

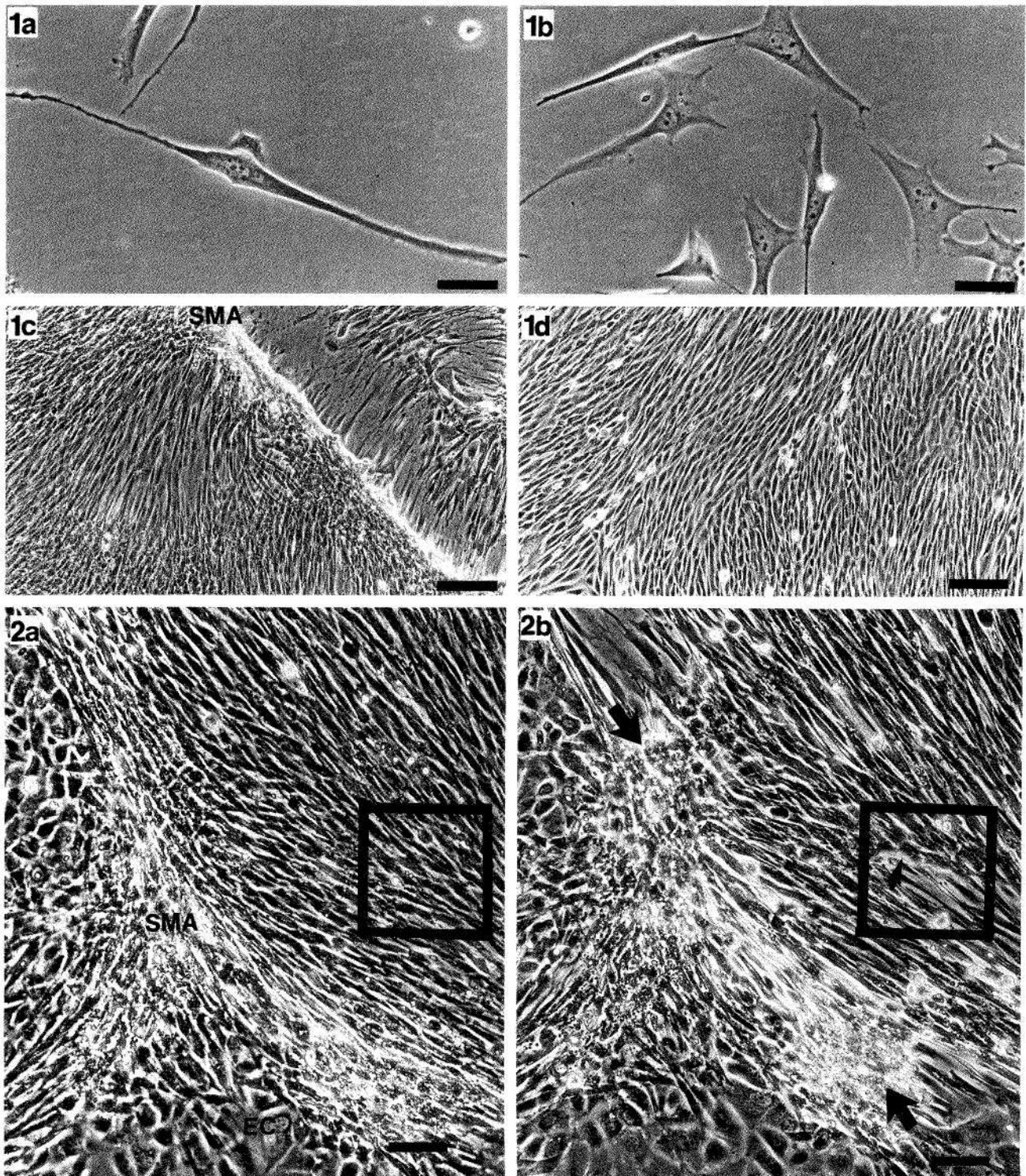
Thirty-minute tracheal digests obtained from guinea-pigs weighing 250 g yield approximately 1000 morphologically differentiated SMCs/600 mg (wet weight) tissue along with small epithelial colonies ranging from 10–100 cells each. Animals weighing 600 g, however, yield less than 100 SMCs/600 mg (wet weight) tissue with epithelial colonies of 100–200 cells each. These differences in yield are presumably due to increased amounts of connective tissue and/or decreased SMC viability in older animals. Longer digests (1–3 h) produce cultures that are rapidly overgrown by an unidentified cell type.

Tracheal smooth muscle cells and epithelial cells attach to the flask substratum and are fully spread within 18–36 h after plating. Spindle shaped SMCs with oval nuclei and phase dense cytoplasm (Fig. 1a) are easily distinguished from epithelial cells which have a polygonal shape, large central nucleus and more lucent peripheral cytoplasm. By the second or third day in culture, most of the morphologically differentiated SMCs broaden and become fibroblastoid (modulated) in appearance (Fig. 1b). Also observed during the initial 18–24 h of culture are single cells which are morphologically indistinguishable from modulated SMCs, suggesting that some tracheal SMCs become biosynthetically active very early in primary culture. Consequently, the actual number of SMCs introduced into primary culture, as calculated by the number of morphologically differentiated SMCs, may be underestimated.

Single epithelial cells in primary culture become vacuolized and do not proliferate. However, mitotic SMCs are often observed as early as day 2 after plating. These cells reach confluence in 5 days (250 g guinea-pigs) to 17 days (600 g guinea-pigs) following intense proliferation in the nutrient rich DMEM/F12 combination medium supplemented with 10% FBS. At confluence, trypsinized primary cultures yield between  $2 \times 10^5$  and  $8 \times 10^5$  viable SMCs/digest depending on the number of single cells and density of epithelial colonies in the initial plating. These cell counts represent 95–97% of the total SMCs recovered. Although this first subculture is generally free of epithelial cells, a few single cells occasionally survive trypsinization and repopulating but experience necrosis within two or three days of subculture. Smooth muscle cells remaining in the primary culture flasks continue to proliferate and may be harvested several times before the epithelial colonies become necrotic.

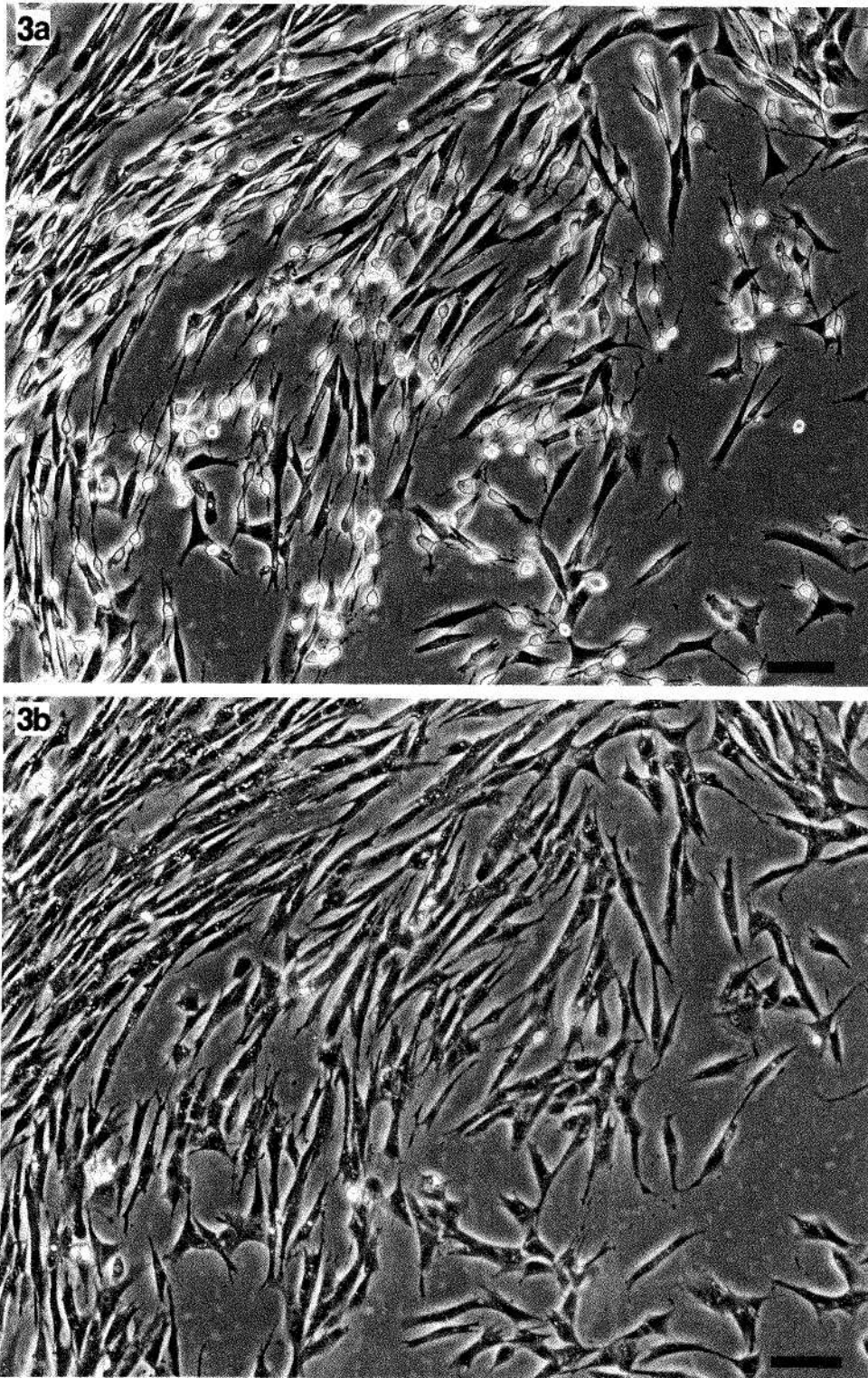
Smooth muscle cells dispersed by the 30-min enzymedigestion proliferate to form parallel arrays of overlapping smooth muscle sheets when plated together with epithelial colonies. When further proliferation on the substratum becomes limited by epithelial outgrowth, smooth muscle cells migrate from the sheets to form ridges and dense muscle aggregates (Fig. 1c). Furthermore, when subcultured, smooth muscle cells migrate from these aggregates to form a monolayer (Fig. 1d) which gives rise to the characteristic "hill and valley" pattern (not shown) typical of smooth muscle (Chamley-Campbell et al. 1979). Unless subcultured within a few days of confluence, muscle aggregates begin





**Fig. 1 a–d.** Morphology of tracheal smooth muscle cells in culture. **a** Spindle shaped tracheal SMCs 2 days in primary culture. Bar = 38  $\mu\text{m}$ ;  $\times 263$ . **b** Modulated tracheal SMCs 3 days in primary culture. Bar = 38  $\mu\text{m}$ ;  $\times 263$ . **c** Formation of ridges and dense smooth muscle aggregates (SMA) 24 days in primary culture. Bar = 152  $\mu\text{m}$ ;  $\times 66$ . **d** Monolayer of subcultured tracheal SMCs derived from SMC aggregates. Bar = 152  $\mu\text{m}$ ;  $\times 66$

**Fig. 2 a, b.** Histamine response of tracheal smooth muscle cells 10 days in primary culture. **a** Epithelial colony (EC) bordered by dense smooth muscle aggregates (SMA). Note rectangular area containing overlapping sheets of SMCs. Bar = 71  $\mu\text{m}$ ;  $\times 131$ . **b** Cells of **Fig. 2a** exposed to histamine  $10^{-6}$  M,  $37^{\circ}\text{C}$ , 20 min. Arrowheads muscle aggregates that have contracted into large foci. Arrow in rectangular region indicates sheets of SMCs that have aggregated. Bar = 71  $\mu\text{m}$ ;  $\times 131$



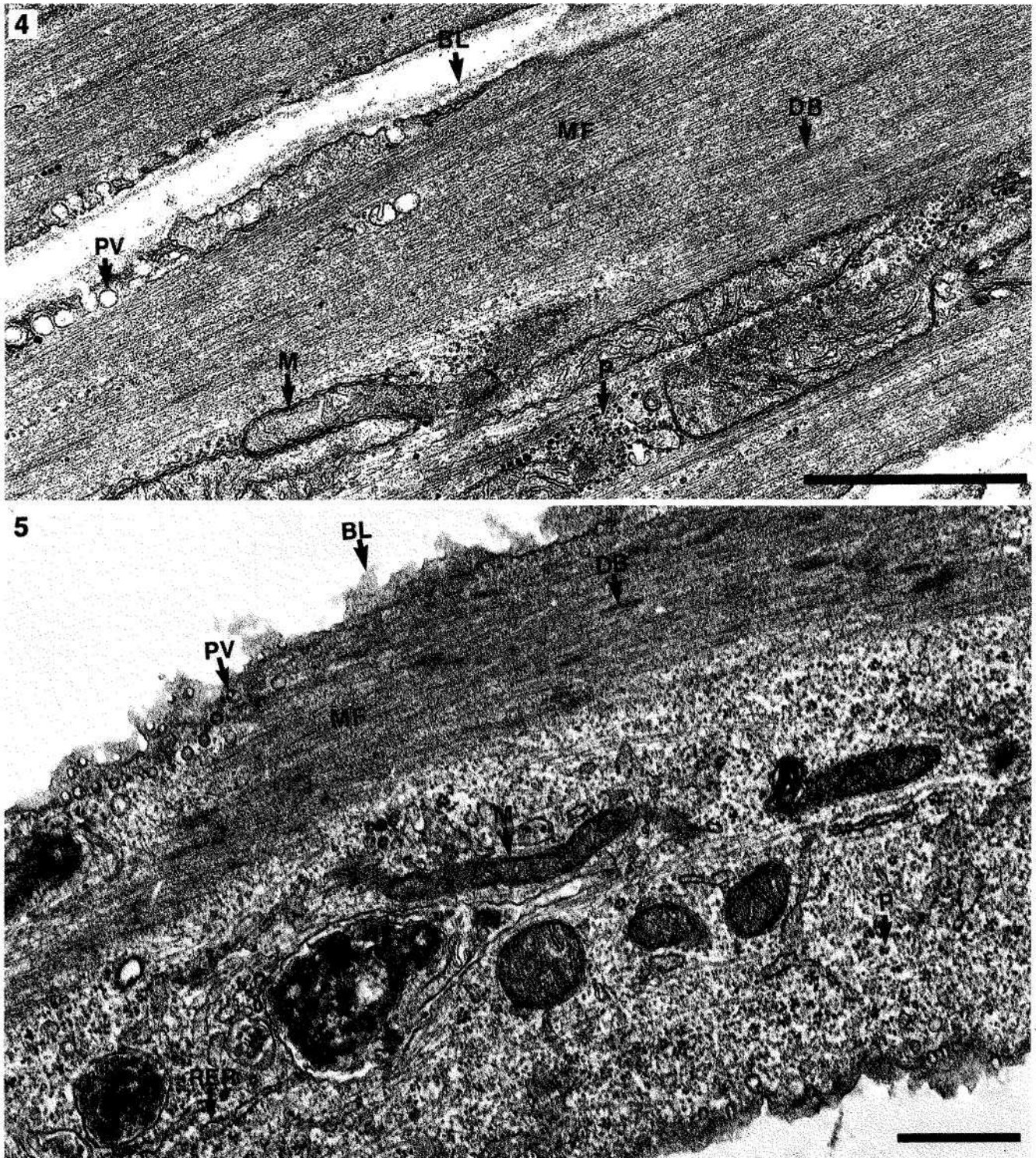
**Fig. 3a, b.** Histamine response of subcultured tracheal SMCs. **a** Response of log phase 2R-42 tracheal SMCs (passage 9) to histamine  $10^{-6}$  M,  $37^{\circ}$  C, 20 min. Bar =  $71\ \mu\text{m}$ ;  $\times 131$ . **b** Relaxation of identical cells 1 h after histamine elution. Bar =  $71\ \mu\text{m}$ ;  $\times 131$ .

to reform although to a much lesser extent than in the primary culture.

Guinea-pig tracheal SMCs in primary culture and subculture contract when exposed to  $10^{-6}$  M histamine. Morphologically, ridges of aggregated SMCs in primary culture contract into large foci while overlapping sheets of SMCs respond by forming smaller aggregates (Fig. 2a, b). Fig. 3a illustrates the typical contractile response of tracheal SMCs

(cell line 2R-42), in mid-log phase of the 9<sup>th</sup> subpassage. Fig. 3b depicts the same cells 1 h after histamine elution with growth medium. Approximately 80% of the cells exhibited some degree of shortening as compared to their morphology without histamine. Log phase and stationary phase cells of the 2R-42 line were challenged with histamine ( $10^{-6}$  M) at subpassages 2, 4, 9, 12 and 20. At least 80% of the cells in both phases of each subpassage contracted.





**Fig. 4.** Electron micrograph of tracheal smooth muscle cells in vivo. **a** Longitudinal section through trachealis muscle. Bar = 1 µm;  $\times 38\,000$ . Myofilaments (MF) and associated dense bodies (DB) occupy most of cytoplasm. Note plasmalemmal vesicles (PV), numerous mitochondria (M), polysomes (P) and basal lamina (BL) surrounding each cell

**Fig. 5.** Electron micrograph of tracheal smooth muscle cells in vitro. Longitudinal section of 2R-42 SMC in 9<sup>th</sup> subpassage (log phase). Bar = 1 µm;  $\times 21\,000$ . Myofilaments (MF), dense bodies (DB), plasmalemmal vesicles (PV), and portions of the basal lamina (BL). Much of cytoplasm occupied by mitochondria (M), polysomes (P) and rough endoplasmic reticulum (RER)

In a total of 8 independent tracheal SMC isolations all have produced subcultures that exhibit a similar contractile response to histamine. While cytoplasmic vacuoles are often observed during cell recovery, the vacuoles persist for less

than 24 h and do not alter cell viability. Neither human nor guinea-pig dermal fibroblasts in primary or subculture were observed to shorten when exposed to histamine.

Definitive features of smooth muscle were identified in

electron micrographs of both undigested trachealis muscle (Fig. 4) and subcultured 2R-42 cells (Fig. 5). Myofilaments and associated dense bodies are observed throughout the cytoplasm of quiescent trachealis smooth muscle, along with plasmalemmal vesicles, several polysomes and a basal lamina surrounding each cell. 2R-42 cells in log phase of growth have numerous plasmalemmal vesicles with myofilaments and dense bodies confined to the cell periphery. Most of the cytoplasm is occupied by polysomes, mitochondria and extensive rough endoplasmic reticulum indicating that these SMCs are synthetically active. The basal lamina surrounding the 2R-42 cells has been disrupted. This was probably due to the mechanical action used to release cells from the flask substratum.

## Discussion

Our studies indicate that a simple, 30-min enzyme digestion releases SMCs from the guinea-pig trachea which retain differentiated function, as determined by histamine response, in long-term proliferative subculture. Electron-microscopic examination of log phase 2R-42 SMCs in the 9<sup>th</sup> subculture reveal definitive characteristics of the SMC contractile apparatus. Histamine challenge continued to elicit a contractile response of at least 80% from both proliferating and nonproliferating cells in this line and seven other SMC lines even after prolonged subculture. To our knowledge, this is the first isolation and characterization of tracheal SMCs which exhibit a histamine-induced contraction while in a proliferative state (log phase) and a non-proliferative state (stationary phase). This observation of retention of differentiated function by tracheal SMCs differs from other reports in that Campbell et al. (1974) and Chamley-Campbell et al. (1979) have shown that proliferating subcultures of vascular smooth muscle cells (vSMCs) do not exhibit a contractile response to vasoactive amines.

It remains unclear, however, whether histamine-responsiveness is a general characteristic of subcultured tracheal SMCs. While Avner et al. (1981) observed that the carbachol response of proliferating primary cultures of canine tracheal SMCs is greatly diminished compared to that of freshly isolated cells, these authors do not report the response of proliferating and nonproliferating subcultured cells. One major difference between this study and our own is the co-culture of tracheal SMCs with epithelial cells.

Cultured endothelial cells have been shown to secrete SMC mitogens (Gajdusek et al. 1980) and to influence low density lipoprotein metabolism (Davies et al. 1985) suggesting that endothelial cells play a role in vSMC growth and differentiation. Epithelial cells may similarly influence the phenotype of tracheal SMCs in vitro. Further studies are needed to determine whether communication between these two cell types regulates the proliferative and contractile properties of SMCs.

Differences in growth-media constituents as well as serum source and preparation may influence tracheal SMC proliferation and differentiation. Concurrently, maintenance of functional histamine receptors and contractile protein associations in proliferating SMCs may be dependent upon a critical combination of nutrients and serum components. Perhaps, the medium used by Avner et al. (1981) (Medium 199 plus 10% heat inactivated fetal calf serum) was not sufficient to maintain the carbachol response of proliferating canine tracheal SMCs in primary culture. In

addition, there may also be intrinsic differences in both in vitro proliferative and contractile properties of tracheal SMCs isolated from different species. Reports that cultured rabbit and calf aortic SMCs differ in elastin secretion (Barone et al. 1985; Schwartz et al. 1980) as well as soluble and filamentous actin (Mogayzel et al. 1985) suggest that such species differences exist between vascular SMCs.

Pure subcultures of functionally differentiated SMCs provide a new system for the study of isolated airway smooth muscle. The current emphasis of in vitro experimentation is on acute chemical and pharmacologic stimulation of airway tissue and the associated alterations in smooth muscle tension. While these studies provide important data on the responsiveness of airway tissue as a whole, they do not allow smooth muscle to be manipulated independently from the neural, epithelial and hemopoietic elements of the tissue. Studies involving the chronic stimulation of airway musculature, a condition more closely simulating chronic asthma, is not feasible due to the limited viability of tissue in vitro. Subcultures of tracheal SMCs which display differentiated function could circumvent this limitation by allowing many cellular responses to be monitored over an extended time period. Analysis of these cultured SMCs may provide more understanding of the biochemical events of induced airway constriction as well as the effect of the epithelium on tracheal SMC growth and differentiation.

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