Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Tracheal matrices, obtained by a detergent-enzymatic method, support *in vitro* the adhesion of chondrocytes and tracheal epithelial cells

Maria Teresa Conconi,[1] Paolo De Coppi,[2] Rosa Di Liddo,[1] Simonetta Vigolo,[1] Giovanni Franco Zanon,[2] Pier Paolo Parnigotto[1] and Gastone Giovanni Nussdorfer[3]

1 Department of Pharmaceutical Sciences, University of Padua, Padua, Italy
2 Division of Pediatric Surgery, Department of Pediatrics, University of Padua, Padua, Italy
3 Department of Human Anatomy and Physiology, University of Padua, Padua, Italy

Keywords
acellular matrix, chondrocytes, *in vitro* model, trachea replacement, tracheal epithelial cells.

Correspondence
Maria Teresa Conconi, Department of Pharmaceutical Sciences, University of Padua, via Marzolo 5, Padua, Italy. Tel.: +390498275714; fax: +390498275366; e-mail: mariateresa.conconi@unipd.it

Received: 3 December 2003
Revised: 16 August 2004
Accepted: 17 December 2004

doi:10.1111/j.1432-2277.2005.00082.x

Summary

Several attemps have been performed to achieve a suitable tracheal replacement for the treatment of different conditions characterized by a lack of sufficient tissue for surgical reconstruction. Actually, tracheal homografts can induce long-term stenosis and their growth potential is not known. Thus, in this work porcine tracheal matrices have been obtained by a detergent-enzymatic method. The treatment decreased the antigenicity of matrices which were able to support the *in vitro* adhesion of both chondrocytes and tracheal epithelial cells. On the contrary, only few cells were observed in tracheal matrices prepared with formalin, Thimerosal, and acetone, suggesting that the long-term stenosis occuring *in vivo* is probably because of an insufficient cell ingrowth. In summary, our results indicate that the detergent-enzymatic method allows us to obtain tracheal matrices which can function as a promising support to achieve an *in vitro* tissue-engineered cell-matrix construct.

Introduction

Different tracheal pathological conditions, such as congenital atresia or stenosis and tumor invasion, are often characterized by a lack of sufficient tissue for surgical reconstruction. Thus, several attempts have been performed to achieve a suitable tracheal replacement. Synthetic materials such as polytetrafluoroethylene, polypropilene mesh, Dacron polyurethane mesh, and silicone rubber, can induce infection, extrusion and stenosis [1]. The latter complication is common to autologous and alloplastic tissues, like cartilage, esophagus, muscle and periosteum [2–5].

Cadaveric human tracheal allografts obtained by a treatment with formalin, Thimerosal, and acetone have been already employed in humans [6]. As these implants do not present viable cells and all major histocompatibility complex (MHC) markers, immunosuppression is not necessary [7]. Nevertheless, tracheal allografts need long-term tracheal stenting when longer segments of trachea with greater circumference have to be replaced. Despite the complete luminal epithelialization of the implanted grafts, there is no live cartilage in the donor trachea [8]. It has been hypothesized [9] that the lack of cell ingrowth could be responsible for the observed stenosis and tracheomalacia. On the contrary, cryopreserved tracheal allografts may represent a valuable alternative, because they do not require immunosuppression and the cellular components remain well preserved after the implantation [9,10]. Moreover, Liu *et al.* [11] showed that tracheal grafts treated with detergent removing epithelium and glands can be used in immunosuppressant-free allotransplantation.

Many lines of evidence pointed out that acellular matrices obtained by a detergent-enzymatic method [12] can be used as bladder [13], urethra [14], and small bowel [15] substitutes, because they allow cell adhesion and growth and are *in vivo* remodeled in a living tissue.

Furthermore, the presence of autologous cells inside the *in vitro* construct represents an important factor which may enhance the biocompatibility of the implant [16]. Over the past years, various biomaterials, such as

PGA, collagen, and calcium alginate gels, have been successfully used in combination with chondrocytes to obtain a tissue-engineered cartilage [17–19]. Moreover, a tracheal substitute composed of chondrocytes and epithelial cells cultured *in vitro* on a degradable scaffold was implanted into a defect previously created in the cervival trachea of nude rats [20]. The animals were able to breathe without mechanical ventilation and the outcomes of surgery revealed that the implants showed a structure resembling the native trachea.

In this preliminary work we have evaluated *in vitro* the antigenicity of porcine tracheal matrices obtained by the Meezan's method [12], and ascertained whether they are able to support *in vitro* the adhesion of both chondrocytes and tracheal epithelial cells.

Materials and Methods

Matrices from pig tracheas

All experiments were performed according to D.L.G.S 116/92 which warrants care of experimental animals in Italy. The research project was approved by the Italian Health Department according to the art. 7 of above mentioned D.L.G.S.

Tracheas, obtained from adult pigs, were stripped of overlying tissue, deprived of trachealis muscle, and rinsed four times in phosphate-buffered saline (PBS) containing 1% antibiotic and antimicotic solution (AF, Sigma Chemical Company, St Louis, MO, USA), and then treated according to Meezan *et al.* [12] or Heberhold *et al.* [6].

The samples were processed as follows: distilled water for 72 h at 4 °C, 4% sodium deoxycholate (Sigma) for 4 h, and 2000 kU DNase-I (Sigma) in 1 M NaCl (Sigma) for 3 h. Matrices were stored in PBS at 4 °C until use. The presence of cellular elements and MHC positive cells were verified histologically (hematoxylin–eosin and immunohistochemical staining) at each cycle. As control, tracheas were treated with 4% formalin in sodium lactate solution for 14 days, 4 g/l Thimerosal (Sigma) in Dulbecco's phosphate buffered saline for 56 days, and stored in acetone for a minimum of 10 days. Samples were washed thoroughly in saline before use.

To quantify the amount of cells still visible after each cycle of the detergent-enzymatic treatment, 10 slides from each specimen was analyzed with an optical microscope (Laborlux S; Leitz, Wetzlar, Germany) and a computer-aided system (software by 'Casti Studio Imaging', Painiga, VE, Italy). The samples were mounted with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) mounting medium for fluorescence with DAPI. The total number of nuclei were visualized at ×250 by fluorescence microscopy. The cell density was expressed as number of nuclei/10^5 μm^2. Mean ± SD were determined for each analysis.

Cell cultures

The auricular cartilage of a newborn pig was minced, and chondrocytes were isolated by digestion with 0.1% collagenase B (Roche, Mannheim, Germany) in Dulbecco's modified Eagle's medium (DMEM; Sigma) at 37 °C for 3 h. Cells were seeded on Petri dishes previously coated with 0.1% gelatin (Sigma), and cultured with DMEM and Nutrient Mixture F12 Ham's (F12; Sigma) containing 10% fetal calf serum (FCS; Biochrom-Seromed, Berlin, Germany), 0.4 μg/ml hydrocortisone, 8 ng/ml cholera toxin, 5 μg/ml insulin, 20 μg/ml adenin, 10 μg/ml tranferrin, 10 μg/ml triiodothyronin, and 1 ng/ml EGF (Sigma).

Tracheal epithelial cells were obtained from the mucosal lining of the trachea tissue by plating small explants on Petri dishes. Cells were cultured with DMEM and Nutrient Mixture F12 Ham's (F12; Sigma) (3:1, v/v) containing 10% FCS, 0.5 μg/ml hydrocortisone, 8 ng/ml cholera toxin, 5 μg/ml insulin, 10 μg/ml tranferrin, 7 μg/ml triiodotyronin, 0.1 ng/ml retinoic acid, and 0.5 ng/ml EGF. Three days after plating cultures were grown in a serum free media. The depth of apical fluid was ≤0.5 mm.

Immunochemistry

The lack of all MHC markers and the presence of b-FGF were evaluated by immunostaining. After each detergent-enzymatic treatment, aliquots of trachea were fixed with 10% neutral buffered formalin for 24 h and embedded in paraffin. Five μm vertical sections were incubated for 30 min at room temperature with PBS containing 10% FCS. Samples were then incubated at 37 °C for 1 h with monoclonal anti-MHC class I OX27, anti-MHC class II OX4, and anti-b-FGF antibodies (Abcam, Cambridge, UK) diluted in 1% FCS–PBS (1:400), and then labeled with avidin–biotin amplified immunoperoxidase method, using the Large Volume Dako LSAB Peroxidase Kit (Dako, Glostrup, Denmark).

Cell ($5 \times 10^4/cm^2$) obtained from primary cultures were seeded in chamber slides (Nunc, Wiesbaden, Germany) and grown for 3–4 days.

Primary tracheal epithelial cells were fixed for 10 min at 4 °C in cold methanol. After washing, fixed cells were incubated for 30 min at room temperature with PBS containing 10% FCS. Samples were then incubated at 37 °C for 1 h with a rabbit anti-mouse cytokeratin 18 antibody (Sigma) diluted in 1% FCS–PBS (1:1000), and then labeled as described above.

Primary chondrocyte cultures were fixed with 2% (w/v) paraformaldehyde, permeabilized using 0.2% Triton X-100 in PBS, washed three times with PBS, and treated with 10% (v/v) normal goat serum in PBS as blocking reagent. The cells were incubated with the primary anti-

body, polyclonal cartilage oligomeric matrix protein (COMP) antiserum diluted in 1% BSA–PBS (1:20) (kindly purchased by Dr M. Paulsson, University of Cologne, Germany) for 60 min, followed by detection with secondary Cγ2-coniugated anti-rabbit IgG antibody (Chemicon International, Temecula, CA, USA). The samples were mounted and examined under a fluorescence microscope (Zeiss Axioplan, Carl Zeiss Oberkochën, Jena, Germany).

Negative controls were carried out by similarly treating matrices and cultures and omitting the primary antibody.

In vitro cultures of tracheal epithelial cells and chondrocytes on homologous matrices

Primary cultures of tracheal epithelial cells or chondrocytes were seeded ($5 \times 10^5/cm^2$) on homologous acellular matrix internal and external side, respectively. The culture surface was about 4 cm^2. The cells were maintained in culture with the media above described (see Cell cultures).

To qualitatively evaluate cell adhesion, at 24 and 48 h after seeding, matrices were fixed with 3% gluteraldehyde (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer (pH 7.2) (Prolabo, Paris, France). After critical point drying and gold sputtering, cultures were examined by a scanning electron microscope (Stereoscan-205 S, Cambridge Instruments, Cambridge, UK). The not reseeded matrices were used as control.

Results

Tracheal matrices

Eighteen cycles of the detergent-enzymatic treatment were not able to completely decellularize trachea specimens (Fig. 1a) in comparison with the not treated tissue (Fig. 1b). Several chondrocytes were still visible inside the cartilage rings, whereas epithelial cells and glands disappeared (Fig. 1c). As shown in Table 1, the samples obtained with higher numbers of cycles (22) were completely decellularized, but they seemed not suitable

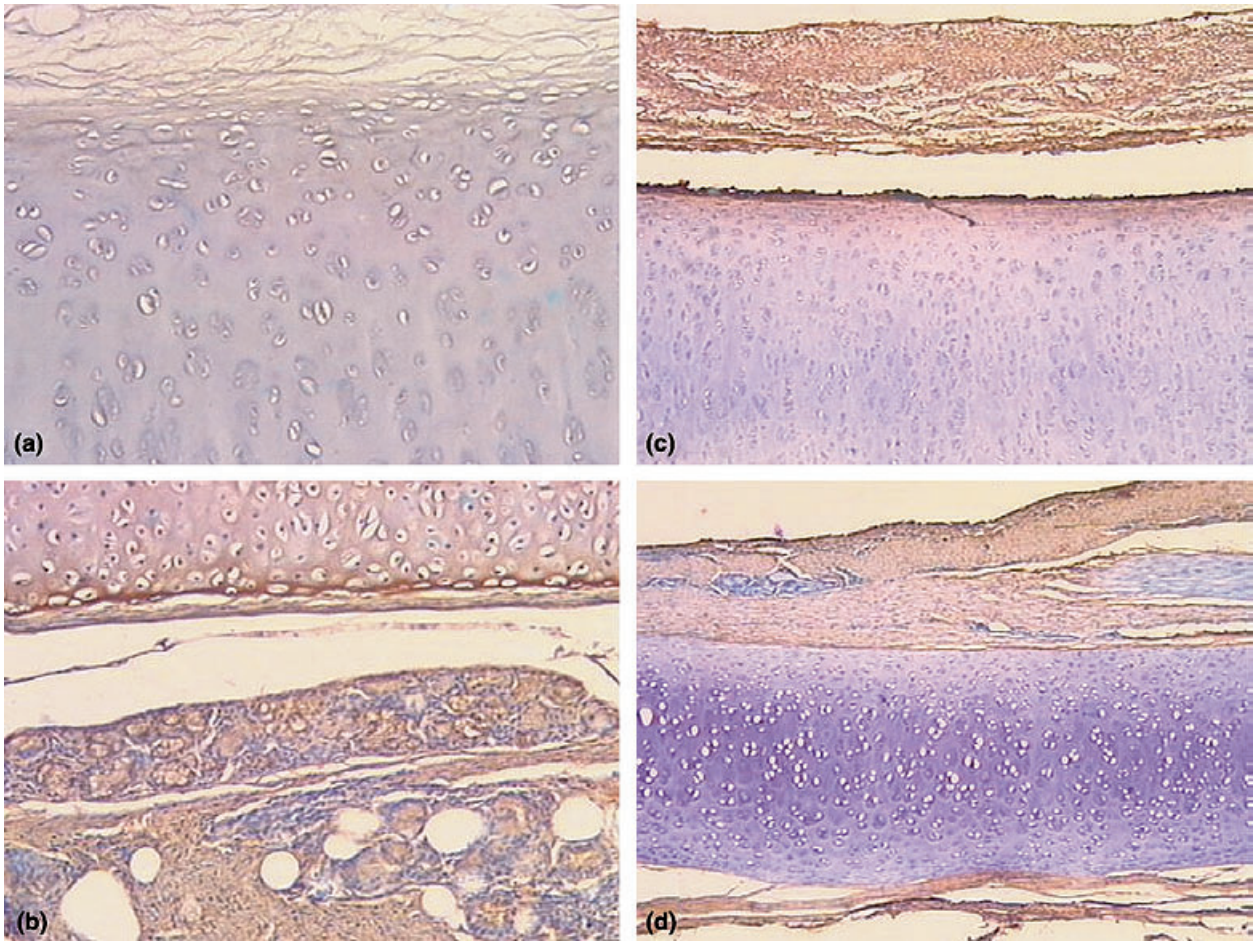

Figure 1 Transversal sections of tracheal matrices after 18 cycles of detergent-enzymatic treatment (a, c) and native trachea (b, d) immunostained with monoclonal anti-MHC class II OX4 (a, b) and anti-b-FGF (c, d) antibodies. (a, b: magnification ×100; c, d: magnification ×40).

Table 1. Amount of nuclei visible in tracheal matrices before and after the detergent-enzymatic treatment.

Number of cycles	Number of nuclei/10^5 μm^2 ± SD
0	311 ± 32
6	220 ± 27
12	123 ± 42
18	29 ± 7
22	0

for surgical purpose because they lose their structural integrity. Eighteen cycles were needed to remove both MHC class I (data not shown) and class II (Fig. 1a) positive cells in the tracheal matrices. So, we decided to use samples treated with 18 cycles for the *in vitro* experiments. Moreover, immunohistochemical staining revealed the presence of b-FGF in both treated (Fig. 1c) and native (Fig. 1d) tracheas. A strong positivity for FGF-2 was observed, mainly located at the connective tissue.

Cell cultures

Cultured chondrocytes displayed the typical polygonal shape (Fig. 2a) and were positively stained by the anti-COMP antibody at day 8 (Fig. 2b).

Tracheal epithelial cells migrated out the explant on the third day, and showed the typical columnar shape with beating cilia (Fig. 3a). In addition, cultured epithelial cells expressed cytokeratin 18 (Fig. 3b).

In vitro cultures of chondrocytes or tracheal epithelial cells on homologous trachea matrices

At 24 h, matrices obtained by the detergent-enzymatic method showed many round chondrocytes (Fig. 4a). At

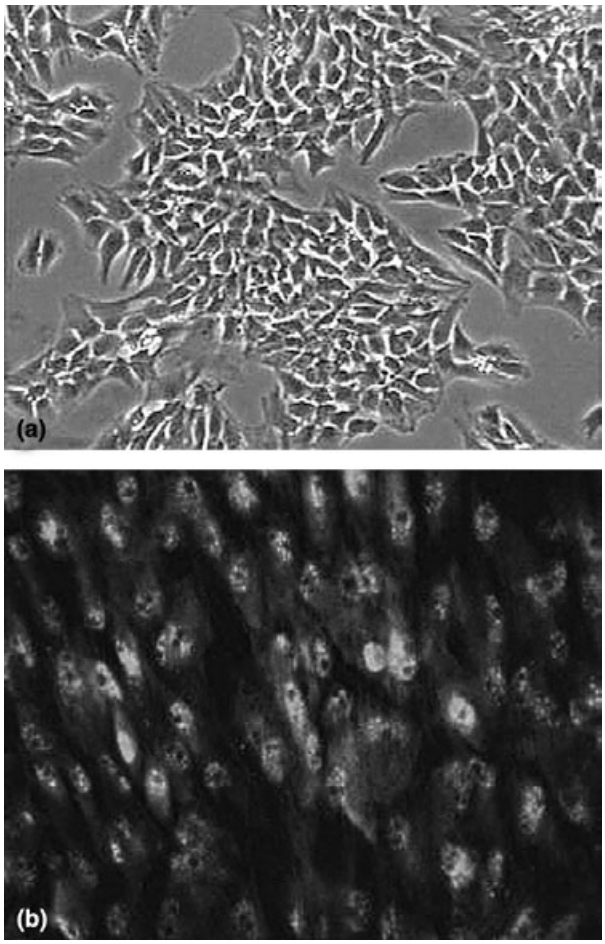

Figure 2 Chondrocyte cultures. (a) Phase-contrast microscopy of cultures 4 days after isolation (magnification ×100); (b) primary cultures immunostained with monoclonal anti-COMP antibody (magnification ×400).

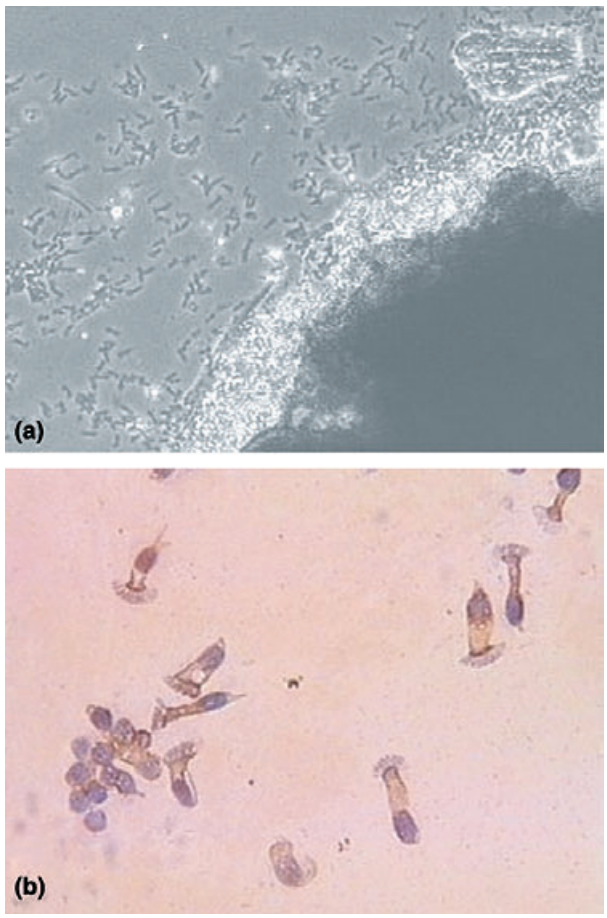

Figure 3 Tracheal epithelial cell cultures. (a) Phase-contrast microscopy of cultures 3 days after isolation (magnification ×50); (b) primary cultures immunostained with monoclonal anti-cytokeratin 18 antibody (magnification ×250).

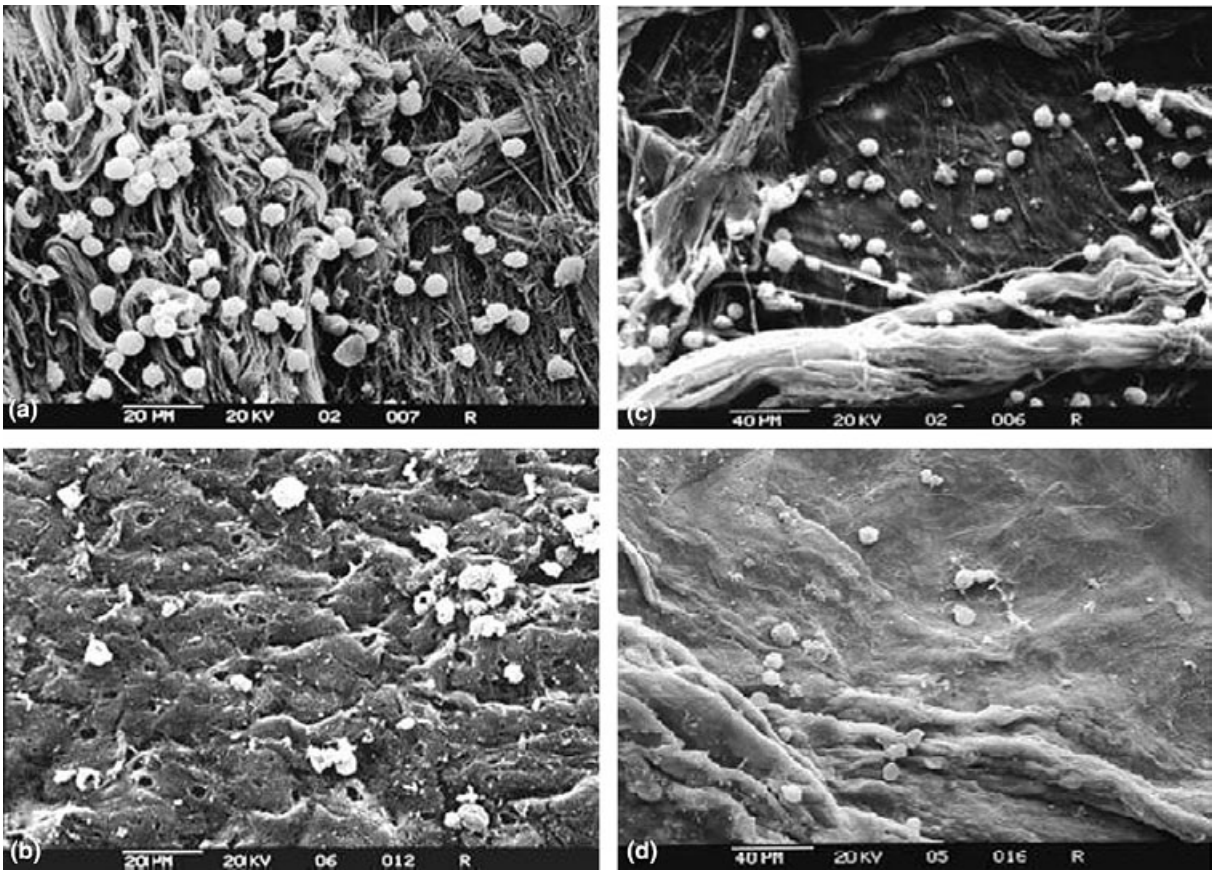

Figure 4 Scanning electron microscopy of tracheal matrices obtained by the detergent-enzymatic method (a, b) and the Heberhold method (c, d) after 24 (a, c) and 48 h (b, d) from chondrocyte seeding.

48 h, cells were more flattened and almost completely covered the matrix surface (Fig. 4b). On the contrary, in tracheas treated with Heberhold's method only few cells were seen at 24 h (Fig. 4c), and after further 24 h their number even decreased (Fig. 4d). Similar results were obtained with tracheal epithelial cells. Ciliated cells attached on matrices treated with detergent and enzyme (Fig. 5a), whereas only few and rounded cells were observed on the controls (Fig. 5b). The surface of not reseeded acellular matrices, used as control, did not present cells (data not shown).

Discussion

In this preliminary work we have demonstrated that tracheal matrices obtained by a detergent-enzymatic method can support *in vitro* the adhesion of chondrocytes and tracheal epithelial cells, thereby suggesting an alternative tissue-engineered approach to the repair or replacement of tracheal defects.

Today most tracheal lesions can be resected and primary reconstruction safely carried out. However, tracheal substitution is needed in the case of very severe stenosis, that cannot be safely corrected by slide tracheoplasty, and of extended tumors in which invasion of larynx or mediastinum does not prevent complete resection with reconstruction of the larynx [21]. According to Belsey [22], a tracheal substitute must have the following requirements: (i) to be a laterally rigid, but longitudinally flexible tube, and (ii) to possess ciliated respiratory epithelium lining. For these reasons, tracheal homograft represents the ideal substitute. However, the growth potential of the tracheal homograft is not known and long-term stenosis can often occur [8]. In the last decades, increasing evidence has enlightened that acellular matrices obtained with the detergent-enzymatic method can stimulate *in vivo* the ingrowth of host cells, matrix formation, and the binding of new cells and matrix to host tissue [13–16]. Those characteristics would eventually prevent the formation of stenosis.

Starting from these considerations, we first demonstrated that 18 cycles of the detergent-enzymatic treatment induces a qualitative reduction in the expression of MHC antigens in porcine tracheal matrices.

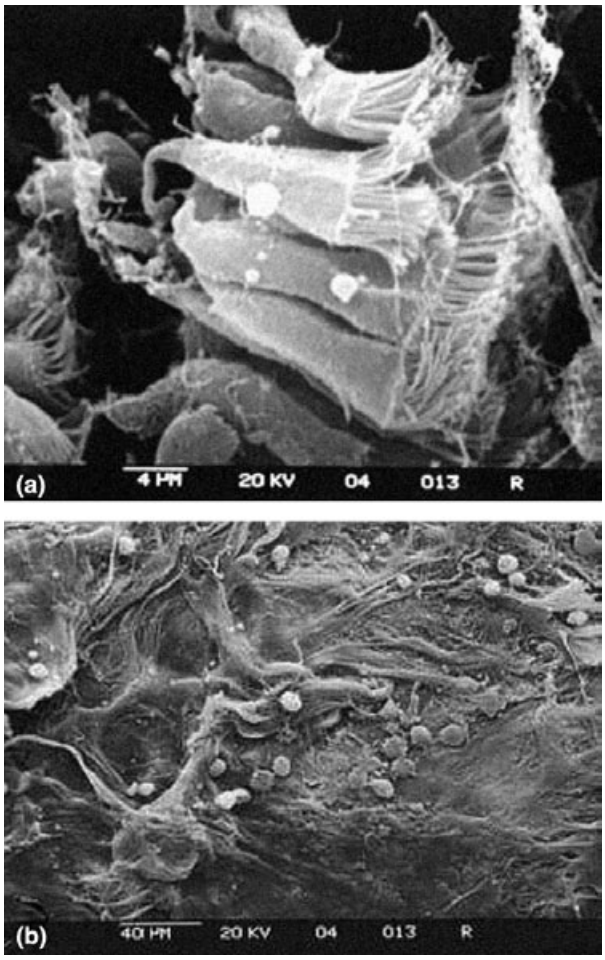

Figure 5 Scanning electron microscopy of tracheal matrices obtained by the detergent-enzymatic method (a) and the Heberhold method (b) after 48 h from epithelial tracheal cells seeding.

Only epithelial cells disappear after treatment, whereas chondrocytes are still visible inside the cartilage. MHC class II and class I positive cells disappear in the treated tracheal matrices. MHC class II antigen is normally expressed by the dendritic cells of the trachea, which function as antigen-presenting cells [23]. Hence, the loss of MHC class II cells could minimize the allograft rejection, because they are essential for the recognition of alloantigen [24]. Moreover, it has been already demonstrated that the immune responses to xenogeneic acellular tissue are of Th2 class, not involving fixation of complement or graft rejection, but rather inducing tolerance [25]. Immunosuppressive therapy is undesirable for many reasons, especially because the principal need for trachea replacement is extensive carcinoma [8].

Nowadays, after *in vivo* implants, a major problem is represented by low levels of cell survival because of a delayed neovascularization [26]. It has been previously reported that with increasing cell density and tissue size, the volumetric oxygen consumption rate in the cell bed increased while the effective substrate diffusivity in the cell bed decreases, resulting in the formation of necrotic areas in the core part of tissues [27]. To solve this problem Tojo *et al.* [10] have successfully used omental flaps to revascularized cryopreserved tracheal grafts. Other approaches can be used for vascularization of bioengineered tissue: incorporation of angiogenic factors in the scaffolds, seeding endothelial cells with other cell types and prevascularization of matrices prior to cell seeding [28]. Immunohistochemical staining revealed the presence of b-FGF [29,30], a well-known angiogenic factor, inside the treated matrices. Further studies will be necessary to ascertain whether the residual b-FGF maintains its angiogenic activity after the detergent-enzymatic treatment.

In the second step of this study we obtained separate cultures of porcine chondrocytes and tracheal epithelial cells. Chondrocytes were derived from the cartilage of newborn porcine ear, cultured in a matrix composed of gelatin, to avoid their dedifferentiation. In fact, when chondrocytes are maintained in monolayer culture, their biosynthetic profile rapidly shifts to a fibroblast-like phenotype [31]. Actually, it has been suggested that the frequent formation of fibrocartilage instead of hyaline cartilage may be the result of the dedifferentiation of chondrocytes occurring in monolayer culture [32]. In our cultures, cells were positive to COMP, one of the major non-collagenous proteins in the cartilage [33]. COMP, together with collagen tipe IX, is more sensitive than other proteins (e.g. collagen type II), to dedifferentiation, thus representing a useful marker of differentiation state of primary chondrocytes [34].

Regarding epithelial cell cultures, we could achieve a ciliated cell differentiation in primary tracheal epithelial cells submerged cultures using depths of apical fluid ≤0.5 mm, according to Ostrowski and Nettesheim [34] which reported that submersion inhibits, but does not prevent *in vitro* ciliogenesis.

Finally, the detergent-enzymatic method allowed us to obtain tracheal matrices which *in vitro* supported adhesion of both chondrocytes and tracheal epithelial cells. On the contrary, only few cells were observed in tracheal matrices prepared by the Heberhold's method, suggesting that the long-term stenosis occurring *in vivo* is probably because of an insufficient cell ingrowth. In fact, even with the extensive washing of these matrices, the chemicals used could remain inside the patches and exert cytotoxic effects and/or modify matrix composition, with ensuing inhibition of cell adhesion and proliferation. Less aggressive treatments, such as repeated freeze–thaw, lyophilization and chemical dehydration, appear more suitable to obtain devitalized cartilage able to maintain cultured chondrocytes [35]. Moreover, it

has been already demonstrated that the detergent-enzymatic method does not alter either the matrix structure or the basement membrane complex [36], and this in turn avoids matrix degradation *in vivo* [37].

In conclusion, our findings indicate that the detergent-enzymatic method allows us to obtain tracheal matrices which can function as a valuable support to achieve an *in vitro* tissue-engineered cell-matrix construct. Further *in vitro* and *in vivo* investigations will be necessary to ascertain whether this approach could represent a promising tool for tracheal replacement. It will be noteworthy to evaluate not only the biomechanical properties of the tracheal matrices, but also the influence of residual chondrocytes after detergent-enzymatic treatment on seeded cells.

References

1. Jacobs JR. Investigations into tracheal prosthetic reconstruction. *Laryngoscope* 1988; **98**: 1239.
2. Cohen FC, Filler RM, Konuma K, Bahoric A, Kent G, Smith C. The successful reconstruction of thoracic tracheal defects with free periosteal gratfs. *J Pediatr Surg* 1985; **20**: 852.
3. Fonkalsrud EW, Sumida S. Tracheal replacement with autologous esophagus for tracheal stricture. *Arch Surg* 1971; **102**: 139.
4. Har-El G, Krespi YP, Goldsher R. The combined use of muscle flaps and alloplasts for tracheal reconstruction. *Arch Otolaryngol Head Neck Surg* 1989; **115**: 1310.
5. Kon M, van den Hooff A. Cartilage tube formation by perichondrium: a new concept for tracheal reconstruction. *Plast Reconstruct Surg* 1983; **72**: 791.
6. Heberhold C, Franz B, Breipohl W. Chemisch-konservierte menschliche trachea als prothesenmaterial zur deckung trachealer defekte. *Laryng Rhinol* 1980; **59**: 453.
7. Jacobs JP, Quintessenza JA, Andrews T, *et al.* Tracheal allograft reconstruction: the total north american and worldwide pediatric experiences. *Ann Thorac Surg* 1999; **68**: 1043.
8. Grillo HC. Tracheal replacement: a critical review. *Ann Thorac Surg* 2002; **73**: 1995.
9. Tanaka H, Maeda K, Okita Y. Transplantation of the cryopreserved tracheal allograft in growing rabbits. *J Ped Surg* 2003; **38**: 1707.
10. Tojo T, Niwaya K, Sawabata N, *et al.* Tracheal replacement with cryopreserved tracheal allograft: experiment in dogs. *Ann Thorac Surg* 1988; **66**: 209.
11. Liu Y, Nakamura T, Yamamoto Y, *et al.* A new tracheal bioartificial organ: evaluation of a tracheal allograft with minimal antigenicity after treatment by detergent. *ASAIO J* 2000; **46**: 536.
12. Meezan E, Hjelle JT, Brendel K. A simple, versatile, non disruptive method for the isolation of morphologically and chemically pure basement membranes from several tissues. *Life Sci* 1975; **17**: 1721.
13. Sutherland RS, Baskin LS, Hayward SW, Cunha GR. Regeneration of bladder urothelium, smooth muscle, blood vessels and nerves into an acellular tissue matrix. *J Urol* 1996; **156**: 571.
14. Parnigotto PP, Gamba PG, Conconi MT, Midrio P. Experimental defect in rabbit uretra repaired with acellular aortic matrix. *Urol Res* 2000; **28**: 46.
15. Parnigotto PP, Marzaro M, Artusi T, Perrino G, Conconi MT. Short bowel syndrome: experimental approach to increase intestinal surface in rats by gastric homologous acellular matrix. *J Pediatr Surg* 2000; **35**: 1304.
16. Marzaro M, Conconi MT, Perin L, *et al.* Autologous satellite cell seeding improves in vivo biocompatibility of homologous muscle acellular matrix implants. *Int J Mol Med* 2002; **10**: 177.
17. Rodriguez A, Cao YL, Ibarra C, *et al.* Characteristic of cartilage engineered from human pediatric auricular cartilage. *Plast Reconstr Surg* 1999; **103**: 1111.
18. Ibarra C, Jannetta C, Vacanti CA, *et al.* Tissue engineered meniscus: a potential new alternative to allogeneic meniscus transplantation. *Transplant Proc* 1997; **29**: 986.
19. Vacanti CA, Kim W, Schloo B, Upton J, Vacanti JP. Joint resurfacing with cartilage grown in situ from cell-polymer structures. *Am J Sports Med* 1994; **22**: 485.
20. Kojima K, Bonassar LJ, Roy AK, Mizuno H, Cortiella J, Vacanti CA. A composite tissue-engineered trachea using sheep nasal chondrocytes and epithelial cells. *FASEB J* 2003; **17**: 823.
21. Mulliken JB, Grollo HC. The limits of tracheal resection with primary anastomosis: further anatomical studies in man. *J Thorac Cardiovasc Surg* 1968; **55**: 418.
22. Belsey R. Resection and reconstruction of the intrathoracic trachea. *Br J Surg* 1950; **38**: 200.
23. Nelson DJ, McMenamin C, Mc William AS, Brenam M, Holt PG. Development of the airway intraepithelial dendritic cell network in rat from class II major histocompatibility(Ia)-negative precursors: differential regulation of Ia expression at different levels of the respiratory tract. *J Exp Med* 1994; **179**: 203.
24. Bujia J, Wilmes E, Hammer C, Kastenbauer E. Tracheal transplantation: demonstration of HLA classII subregion gene products on human trachea. *Acta Otolaryngol* 1990; **110**: 149.
25. Allman AJ, McPherson TB, Badylak SF, Merril LC, Kallakury B, Sheenan C, *et al.* Xenogeneic extracellular matrix grafts elicit a Th2-restricted immune response. *Transplantation* 2001; **71**: 1631.
26. Mooney DJ, Mikos AG. Growing new organs. *Sci Am* 1999; **280**: 60.
27. Sardonini CA, Di Biasio D. An investigation of the diffusion-limited growth of animal cells around single hollow fibers. *Biotech Bioeng* 1992; **40**: 1233.

28. Nomi M, Atala A, De Coppi P, Soker S. Principals of neovascularization for tissue engineering. *Mol Aspects Med* 2002; **23**: 463.
29. Ribatti D, Urbinati C, Nico B, Rusnati M, Roncali L, Presta M. Endogenous basic fibroblast growth factor is implicated in the vascularization of the chick embryo chorioallantoic membrane. *Dev Biol* 1995; **170**: 39.
30. Abbott J, Holtzer H. The Loss of Phenotypic Traits by Differentiated Cells. III The Reversible Behaviour of Chondrocytes in Primary Cultures. *J Cell Biol* 1966; **28**: 473.
31. Nehrer S, Spector M, Minas T. Histologic analysis of tissue after failed cartilage repair procedures. *Clin Orthop* 1999; **365**: 149.
32. Hedbom, E, Antonsson P, Hjerpe A, *et al.* Cartilage matrix proteins. An acidic oligomeric protein (COMP) detected only in cartilage. *J Biol Chem* 1992; **267**: 6132.
33. Zauche F, Dinser R, Maurer P, Paulsson M. Cartilage oligomeric matrix protein (COMP) and collagen IX are sensitive markers for the differentiation state of articular primary chondrocytes. *Biochem J* 2001; **358**: 17.
34. Ostrowski LE, Nettesheim P. Inhibition of ciliated cell differentiation by fluid submersion. *Exp Lung Res* 1995; **21**: 957.
35. Kelley TF, Sutton FM, Wallace VP, Wong BJ. Chondrocyte repopulation of allograft cartilage: a preliminary investigation and strategy for developing cartilage matrices for reconstruction. *Otolaryngol Head Neck Surg* 2002; **127**: 265.
36. Livesey SA, Herndon DN, Hollyoak MA, Atkinson YH, Nag A. Transplanted acellular allograft dermal matrix. *Transplantation* 1995; **60**: 1.
37. Burge SM, Dawber RPR. Hair follicle destruction and regeneration in guinea pig skin after cutaneous freeze injury. *Cryobiology* 1990; **27**: 153.