From grunts to words: experiments in laryngeal transplantation

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ABSTRACT

INTRODUCTION Laryngeal transplantation remains an increasingly viable option for patients with irreversible disease or damage to the larynx. Successful organ transplantation relies on minimising surgical, ischaemic and immunological insults. The inherent immunogenicity of an organ is dependent on the amount of immunologically active cells within it. The presence of immunologically active cells within non-transplanted NIH-minipigs was investigated and an *in vivo* laryngeal transplant model was developed.

MATERIALS AND METHODS Quantitative, multiple-colour immunofluorescence using pig-specific monoclonal antibodies was used to assess the normal immunological architecture and the short-term immunological changes associated with 3 h of cold ischaemia and 8 h of reperfusion in an MHC-matched animal model.

RESULTS AND CONCLUSIONS There is a complex immunological architecture within the non-transplanted, healthy pig larynx. In addition, an *in vivo* laryngeal transplant model was developed that allowed successful perfusion for 8 h post transplantation. There were significant changes in cell numbers within different anatomical subsites of the larynx. However, the biological significance remains debatable in view of the large range of cell numbers both within and between individual animals.

KEYWORDS

Laryngeal transplant – NIH-minipigs – Immunology

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John Hunter (1728–1793) is considered to be the most influential surgeon and anatomist of the 18th century. Hunter did not transplant laryngeal allografts. However, he did document transplanting a cockerel's spur into its comb, the testes of a cockerel into a hen, and human teeth into the highly vascularised comb of a cockerel! As such, he demonstrated autotransplantation, allotransplantation and xenotransplantation respectively. Many consider him to be the first to coin the phrase 'transplant'.¹

For many, transplantation in the 18th century was inconceivable. To some, the concept of laryngeal transplantation in the 21st century remains obscure. So why are researchers around the world focusing on replacing one human larynx with another human larynx? A laryngectomy is performed as a result of disease or irreversible damage to the larynx. It was first described by Billroth in the 1870s and the procedure remains very much the same today as it did then. At operation, the larynx is removed in continuation with the distal pharynx and proximal trachea. The remaining trachea is secured to the anterior neck wall as an end

tracheostome. The pharynx is closed forming a continuous tube from the mouth to the oesophagus.

There are a number of devastating personal and physiological outcomes associated with a laryngectomy. These range from voice loss, both in close proximity, and over distance using the telephone. Without a larynx, kissing, coughing, straining, laughing and crying are all impaired or impossible. Importantly, a laryngectomy also removes a vital part of the upper respiratory tract, the pathway normally used for warming and humidification of gases. In addition, as the olfactory nerves are bypassed by the tracheostome, both smell and taste are diminished.

Conventional methods of voice and swallowing replacement after total laryngectomy unfortunately continue to be sub-optimal. By contrast, the striking vocal, deglutition and quality-of-life accomplishments of the one human laryngeal transplant recipient² show what may be possible if research continues and transplantation becomes a viable clinical option.

If the larynx were an inert piece of scaffold, simply there to provide mechanical protection and allow phonation, then

A

B

Figure 1. Expression of MHC class II and lymphocyte cell antigens in the pig laryngeal mucosa. (A) Blue, nuclear material stained with DAPI; green, MHC class II; red, CD45. (B) Blue, nuclear material stained with DAPI; green, MHC class II; red CD3 T cells. The close association between the 'dendrites' of the MHC class II cells and the CD3 T-cells have been arrowed.

transplanting it would purely be a technical exercise. However, if the larynx, in common with all other mucosal surfaces investigated to date, contained an immunological architecture, then the technical 'plumbing-in' of the graft would only be the first step. Mucosal surfaces, including the lungs and intestine, contain an abundance of immunologically active cells which have a direct effect on the amount of immunosuppression required and, ultimately, graft survival.^{3,4}

Very little scientific data existed regarding laryngeal immunology. Initially, we investigated the mucosal architecture of normal, healthy non-transplanted larynxes using NIH-minipigs as our model. Once we had evaluated the typical architecture, we developed an *in vivo* laryngeal transplant model and assessed the short-term immunological changes associated with 3 h of cold ischaemia and 8 h of reperfusion in an MHC-matched animal. Outlines of the results are presented.

Materials and Methods

Animals

All pigs were NIH-minipigs.⁵ Both anaesthetic and operative details have previously been described by our group.^{6,7} These studies were approved by the appropriate local and national ethical boards (UK Home Office PPL 30/1786).

Pre-retrieval biopsies (donor)

After anaesthesia, laryngeal biopsies were taken from the laryngeal sub-sites cranial and caudal to the vocal cords ('supraglottis' and 'subglottis'). This separation is important as these sites are different anatomically and functionally. Biopsy tissue was covered in OCT and snap-frozen in isopentane cooled over liquid nitrogen as previously described.⁸ The cores were stored at -70°C until further processing.

Operative details (donor)

In brief, the larynx and trachea were identified and supplying vessels isolated. Specifically, the axillary artery was isolated with a vasilop (Bard, UK). The venous vascular bundle was slooped independently, but not tied, to prevent venous obstruction and resulting limb oedema. The nerves of the brachial plexus were identified, but not cut until after the pig had been killed, in order to minimise the physiological response to the nociceptive stimuli. Soft tissue dissection was continued cranially to the level of the hyoid bone. There, the common carotid artery was identified and slooped, retaining the fine arterial supply to cranial and caudal larynx. The internal jugular vein and vago-sympathetic trunk were identified and slooped together.

The sternum was opened in the midline allowing access to the great vessels within the thorax. The aorta was isolated and the animal given 5000 IU of intravenous heparin (CP Pharmaceuticals, UK). Five minutes following the heparin administration, the animal was killed with pentobarbitone

(100 mg/kg; Rhone Merieux Ltd, UK). Arterial clamps were placed across the proximal ascending aorta and the proximal descending aorta. The graft was perfused under gravity for 10 min via an aortic root cannula (14-G, Medtronic Inc., Minneapolis, USA) with 900 ml of University of Wisconsin solution at 4°C (Viaspan®; Dupont Pharma, UK).

During this time, sloops on the graft vessels were tied in order to isolate the graft. An ice pack was placed over the graft to keep it cool. Cutting diathermy and ties were used to remove the graft from the carcass. Once the graft was free from the animal it was placed in an Alden intestinal bag (330 x 254 mm, Rush, UK), filled with 100 ml of cold University of Wisconsin solution and stored on ice to complete the 3-hour cold ischaemic time.

Operative details (recipient)

Following cold ischaemia, the graft was reperfused by the recipient animal. Prior to vessel anastomosis, the recipient animal was heparinised as described. The recipient proximal common carotid artery was anastomosed end-to-end to the graft's proximal subclavian artery. The distal graft subclavian vessel was anastomosed end-to-end to the distal common carotid recipient artery. Following the second anastomosis, all the arterial clamps were removed, cold ischaemia ended, and the time of graft reperfusion was recorded. Venous flow was identified leaving the graft through the cut end of the superior vena cava. The venous anastomosis was end-to-side, the end of the superior vena cava of the graft to the side of the jugular venous confluence.

Following 8 h of reperfusion, the graft was removed and laryngeal tissue taken from the supra- and sub-glottis. This tissue was also covered in OCT, snap-frozen in isopentane cooled over liquid nitrogen and stored at -70°C until further processing. The animal was then killed with pentobarbitone (100 mg/kg).

Immunohistology

The samples were processed and prepared for multiple colour immunofluorescence as previously described.⁸ Briefly, frozen sections (4–6 μm) were incubated with a series of solutions including 5% goat and 5% pig serum, optimally diluted primary antibody and secondary, fluorochrome- or biotin-conjugated isotype-specific anti-mouse antibody (Southern Biotechnology Associates Inc., USA). Tertiary staining was achieved by incubation with AMCA Avidin D (Vector laboratories Inc., USA). Nuclear material was stained using 4,6-diamidino-2-phenylindole (DAPI, 0.08 mg/ml; Sigma).

Cells were visualised using a combined blue, green and red filter, using a Leica DMR epifluorescence microscope (Leica, Milton Keynes, UK) and images captured using a high sensitivity colour camera (Colour Coolview; Photonic Science, UK) and image analysis software (Image Pro Plus; Media Cybernetics®, Silver Spring, MD, USA). For each biopsy, approximately 5 fields were captured. Cells were

manually tagged in each image according to their subset using the manual tagging facility in Image Pro Plus. The count was repeated several times by the same operator on separate occasion and mean values obtained. Leukocytes in the epithelium and in the lamina propria were counted separately and the results expressed as cells/mm².

Pseudoconfocal pictures were produced by capturing 20–30 images at different focal depths from a 4–6 µm tissue section. Each image was blurred in turn by image analysis software (Image Proplus; Media Cybernetics®). The blurred image was then subtracted from the original image, leaving just the in-focus information. The in-focus information from the 20–30 images was compiled to create one picture.

Results

Histologically, the pig larynx is made up of stratified squamous epithelium above and including the vocal cords,

although the level of stratification differs between these anatomical subsites (data not shown, see Barker *et al.*⁹). Below the cords, the epithelial type is respiratory in nature, pseudostratified columnar, and is continuous with the mucosa of the trachea (data not shown, see Barker *et al.*⁹).

The non-transplanted pig larynx contained an abundance of immunologically active cells (Fig. 1A,B). Using pseudoconfocal microscopy, the intimate relationship between MHC class II positive cells with dendritic morphology and CD3 positive cells was identified (Fig. 1B). Discrete anatomical subsites of the larynx contained different densities of immunologically active cells.⁹ The glottis was consistently almost devoid of cells (data not shown).

Four animals received MHC-matched grafts with a cold ischaemic time of 3 h and were perfused for 8 h in the recipient animal. All animals survived and the grafts were healthy following reperfusion. Biopsies were quantitatively analysed using multiple-colour immunofluorescence as

Table 1 Mean (± SD) of the densities of the different cell types (cells/mm²) in the pig laryngeal mucosa

	Epithelium		Lamina propria	
	Induction	8 h	Induction	8 h
CD172	66 (48)	240 (175)	28 (33)	453 (390)
CD172 CD163 MHC II	299 (361)	573 (290)	117 (76)	768 (602)
MHCII CD172	160 (195)	217 (263)	194 (134)	953 (667)
MHCII	73 (86)	155 (181)	39 (31)	190 (61)
MHC II	403 (475)	795 (328)	238 (79)	540 (164)
CD14 CD16	213 (184)	491 (311)	168 (109)	498 (214)
CD14 CD16 MHCII	19 (23)	104 (109)	124 (27)	204 (105)
CD4	116 (158)	151 (177)	55 (40)	135 (54)
CD4 CD25	178 (232)	43 (33)	205 (272)	64 (47)
CD4 CD45RC	6 (11)	17 (22)	2 (4)	12 (7)
CD4 CD25 CD45RC	0	0	0	1 (2)
CD8	191 (177)	366 (125)	182 (149)	176 (78)
CD8 CD25	111 (88)	61 (33)	16 (20)	39 (18)
CD8 CD45RC	0	89 (60)	5 (6)	57 (16)
CD8 CD25 CD45RC	0	0	0	0.44 (1)
CD4	128 (175)	268 (69)	272 (112)	335 (180)
CD8	154 (185)	481 (150)	205 (152)	210 (102)
δγ	74 (96)	161 (130)	21 (41)	87 (40)
CD8 δγ	3 (5)	63 (65)	2 (5)	23 (11)

The numbers are calculated from 4 laryngeal allografts at induction of anaesthesia of the retrieval operation and following both 3 h of cold ischaemia and 8 h of reperfusion.

described. The cell numbers before and after transplantation were recorded. The monoclonal antibody combinations used to quantify both myeloid and lymphoid cell lines are shown in Table 1. This table also demonstrates an example of the change in cells numbers observed before and after transplantation. Graphical representation of this change is shown in Figure 2.

Discussion

In evolutionary terms, the larynx first appeared in the lungfish as a muscular sphincter at the floor of the pharynx. This primitive larynx was progressively modified to contain subsequently both dilator fibres and cartilage. Therefore, the principal purpose of the larynx was not for phonation, but as a protecting sphincter for the lower airways. This protective role remains crucial for airway competence and without it, aspiration and infection result. However, in addition to the important function of phonation and protection of the lower airways, the larynx may also represent an important mucosal site immunologically.⁹⁻¹¹ Despite being considered as an inert conduit, it does have a mucosal surface and mucosal surfaces contain high levels of immunologically active cells in the healthy, non-inflamed state.^{3,4,9-11}

The immunological cells within the upper respiratory tract may have an array of diverse and essential functions including a role in infection, allergy and immunosurveillance. Infections within the larynx are often compartmentalised into distinct anatomical subsites.¹²⁻¹⁴ The sharp demarcation of oedema associated with epiglottitis and supraglottitis may represent changes in mucosal defence mechanisms in each discrete subsite. Similarly, localised allergic responses may be a reflection of the underlying immune system determining between mounting an active immune response or maintaining tolerance. Finally, the

concept of 'immunosurveillance' was introduced by Burnet¹⁵ in 1967 to suggest that the immune system engaged in continued surveillance for, and destruction of, potentially neoplastic cells, although the concept of such a mechanism had been proposed a number of years previously.¹⁶ The frequency of tumour occurrence is not uniform throughout the upper respiratory tract. The supraglottis and glottis are affected with a prevalence of 1.3 and 2.3 per 100,000 population, compared to the subglottis and trachea, both of which are involved at a rate of less than 0.04 per 100 000 population.¹⁷ Interestingly, there are significant variations in distribution dependent on geography. Clearly, numerous factors will impact on the initiation and promotion of tumours in the upper respiratory tract and there are many hypotheses regarding the non-uniform distribution of laryngeal cancer. Although both carcinogen exposure and embryological derivation may play an important role in carcinogenesis, we have previously proposed a role, in part, for immunosurveillance and have published data to support this concept.⁹ Other studies have demonstrated an association between high dendritic cell numbers in man and prognosis after resection of squamous cell carcinoma.¹⁸ This adds to evidence that immunosurveillance is an important defence against laryngeal cancer, such as the association with HPV infection.¹⁹ There is a complex relationship between tumour prognosis and inflammatory cytokines²⁰ and the introduction of cytokines can significantly alter tumour response (e.g. IL-2²¹).

There is no good surgical, medical or prosthetic solution to the problems faced by those with a larynx whose function is irreversibly damaged by tumour or trauma. For more than 10 years, the impetus for research into laryngeal transplantation has increased progressively. The biggest milestone in this field was the world's first true laryngeal transplant performed in Cleveland, Ohio in 1998. Although the recipient's graft continues to function well, in many respects more than 8 years later, it has highlighted the outstanding obstacles to full-scale clinical trials.

Our initial results showed an abundance of immunologically active cells within the non-transplanted healthy larynx (Fig. 1A,B).⁹ It is known that the immunogenicity of transplanted tissue correlates with the content of specialised antigen-presenting cells within it.²² Hornick and Lechler²² presented evidence that the early immunogenicity of an allogeneic tissue is dependent on the number of professional antigen-presenting cells (MHC class II positive cells: able to provide 'signal 1' and 'signal 2') to the recipient T-cells. The results from other studies on the immunological architecture of mucosal tissues, together with results in the larynx, suggest that this is attributable to the high prevalence of antigen-presenting cells throughout mucosal tissues. Therefore, transplantation of the larynx would invoke rejection without appropriate MHC-matching

or therapeutic manipulation. Our opening question was directed towards the early immunological reaction in an MHC-matched animal.

The grafts were viable after 8 h of reperfusion. There were significant changes in cell numbers within each subsite of the larynx (Table 1, Fig. 2).⁷ The direction of change in cell numbers was not consistent between subsites.⁷ As an overview, cells within the supraglottis decreased during the time course of the experiment and those in the subglottis increased (Fig. 2).⁷ We have hypothesised a number of factors that may have influenced the anatomical subsites independently; these include, epithelial type, blood vessel and lymphatic vessel density.⁷ Interestingly, despite the significant changes in the cell numbers, the magnitude of changes were less than the large normal inter- and intra-pig variation in cell counts previously described.^{7,9} The significance of injury in a laryngeal allograft is open to question and further studies are required. Finally, there are other questions that are being addressed before laryngeal transplantation enters clinical trials; these include organ retrieval, immunomodulation and functional re-innervation.

Conclusions

The larynx is an immunologically active organ probably involved in complex immunological decisions including immunosurveillance, tolerance and active immunity. We performed laryngeal transplants in a large animal model to investigate the short-term results of laryngeal transplantation. In the MHC-matched animal, the graft survived to 8 h. Longer term experiments are now required for further evaluation.

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