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MAIN ARTICLE

Tacrolimus enhances the recovery of normal laryngeal muscle fibre distribution after reinnervation

P GORPHE[1,2,3], N GUEROUT[2], M BIRCHALL[4], G TERENGHI[5], J-P MARIE[1,2]

[1]Otorhinolaryngology and Head and Neck Surgery Department, Rouen University Hospital, [2]Experimental Surgery Laboratory, Faculty of Medicine, Rouen University, [3]Otorhinolaryngology and Head and Neck Surgery Department, Hôpital Avicenne, Bobigny, France, [4]UCL Ear Hospital, University College London, and [5]Blond McIndoe Laboratories, Tissue Injury and Repair Group, School of Medicine, University of Manchester, UK

Abstract

Objectives: To assess the recovery of various muscle fibre types in the posterior cricoarytenoid muscle after laryngeal reinnervation in the rat, and to determine the influence of tacrolimus on this process.

Methods: Four groups of rats underwent resection and anastomosis of the left vagus nerve, and were administered either tacrolimus at a low dose or an immunosuppressive dose, or cyclosporin A at a low dose or an immunosuppressive dose. A fifth group received surgery alone, and a sixth group received neither surgery nor drug treatment (healthy group). Muscles were removed for immunohistochemical analysis 45 days after surgery.

Results: There was no difference in the proportion of types 1, 2a and 2b muscle fibres, comparing the immunosuppressive tacrolimus group and the healthy group, whereas there were fewer type 1 fibres in the group receiving surgery alone, compared with the healthy group (7 *vs* 12.1 per cent, respectively; $p = 0.0303$).

Conclusion: Tacrolimus enhanced the recovery of normal laryngeal muscle fibres after reinnervation in the rat, indicating a possible role in laryngeal transplantation.

Key words: Tacrolimus; Laryngeal Reinnervation; Calcineurin Inhibitors; Muscle Fibers

Introduction

The intrinsic muscles of the larynx permit the normal laryngeal functions of breathing, swallowing without aspiration, and speech. Inspiration requires opening of the glottis by the abductor muscles. The posterior cricoarytenoid muscles are the main abductors of the glottis and are innervated by the inferior laryngeal branch of the recurrent laryngeal nerve. The reinnervation of these muscles after traumatic or surgical injury of both recurrent laryngeal nerves is essential to avoid permanent glottic closure and tracheostomy. Muscle function depends on the distribution of slow and fast muscle fibres. The correlation between reinnervation and changes in slow and fast muscle fibre distribution is understood in skeletal muscles, but not in the intrinsic laryngeal muscles. Therefore, laryngeal reinnervation with functional recovery remains elusive, and of preliminary consideration for laryngeal transplantation studies.[1–3]

Cyclosporin A and tacrolimus are two calcineurin inhibitors used to prevent allograft rejection following transplant surgery. Tacrolimus influences muscle recovery by modifying reinnervation and, through the calcineurin pathway, muscle fibre distribution. Since the original study by Gold and Villafranca in 2003,[4] the neuroprotective and neuroregenerative effects of tacrolimus on peripheral nerves have been further demonstrated in several publications.[5–9] In addition, calcineurin has been shown to be involved in the distribution of slow and fast muscle fibres in skeletal muscle by the control of myosin heavy chain gene expression. Considering these two mechanisms, the use of tacrolimus as an immunosuppressant in functional transplantations should have a positive influence on laryngeal muscles functions recovery and muscle fibre distribution after reinnervation.[10]

This study aimed to assess the recovery of muscle fibres in the posterior cricoarytenoid muscle after reinnervation in the rat, and to assess the influence of calcineurin inhibitors, especially tacrolimus, on this process.

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Materials and methods

Drugs and surgery

All procedures were approved by the Regional Ethics Committee of Upper Normandy (protocol number 76.A.21).

We used 36 3-week-old, male Wistar rats, divided into 6 groups of 6 rats each. The groups are detailed in Table I.

Surgical procedures were performed in the Experimental Surgery Laboratory of the Faculty of Medicine, Rouen University, France. Surgical resection and anastomosis of the left vagus nerve was performed on each rat from groups A, B, C, D and F. Either cyclosporin A or tacrolimus was given orally every day for 45 days to those animals in groups A, B, C and D. Rats from group E, the healthy control group, received no surgical or pharmaceutical intervention.

On day 45 after surgery, all rats were euthanised. A blood sample was taken to verify the drug concentration, and the left posterior cricoarytenoid muscle was dissected out and sent for immunohistochemical analysis.

There were no intra-operative deaths. One rat from group B (immunosuppressive cyclosporin A) was found to have a rupture of the anastomosis secondary to wound infection, and was excluded from the study.

All drug plasma concentrations in each group were in accordance with the drug kinetics reported in the literature. As expected, the plasma levels of rats receiving 1 mg/kg/day tacrolimus or 2 mg/kg/day cyclosporin A were not within human immunosuppressive levels, whilst the plasma levels of rats receiving 8 mg/kg/day tacrolimus or 15 mg/kg/day cyclosporin A were within immunosuppressive levels.

Immunohistochemistry

The histological procedures were performed in the Blond McIndoe Plastic and Reconstructive Surgery Laboratory of the Manchester University Medical School, UK.

Muscles were frozen in liquid nitrogen and then cut in a cryostat at −23°C to give four thin transverse sections (15 μm) for each muscle.

After a 30-minute bath in 0.2 per cent Triton X-100 and 1 M phosphate-buffered saline then a 5-minute bath in 1 M phosphate-buffered saline, the slides were incubated for 1 hour in 5 per cent normal goat serum in an antibody-diluent solution (phosphate-buffered saline plus 0.1 per cent sodium azide plus 0.1 per cent bovine serum albumin plus 0.03 per cent Triton X-100). The slides were then incubated for 1 hour in the first antibody solution: 0.5 per cent anti-laminin antibody (Sigma, St-Louis, MO, USA), with either 5 per cent HB287 anti-myosin heavy chain type 1 antibody (American Type Culture Collection, Manassas, Virginia, USA), or 5 per cent American Type Culture Collection HB277 anti-myosin heavy chain type 2a antibody, or 10 per cent American Type Culture Collection HB283 anti-myosin heavy chain type 2b antibody. After three 10-minute baths in 1 M phosphate-buffered saline, the slides were incubated for 1 hour in darkness with the second antibody solution: 0.5 per cent CY3 anti-mouse, with 1 per cent fluorescein isothiocyanate immunoglobulin G goat anti-rabbit (FI-1000; Vector Laboratories, Burlingame, California, USA), diluted in antibody-diluent solution (phosphate-buffered saline plus 0.1 per cent sodium azide plus 0.1 per cent bovine serum albumin plus 0.03 per cent Triton X-100).

After three 10-minute baths in 1 M phosphate-buffered saline, the slides were mounted with Vectashield H-1000 (Vector Laboratories, Peterborough, UK) for immunofluorescence microscopy. Whole muscle sections were photographed under immunofluorescence microscopy, using an Olympus digital camera (Tokyo, Japan) and the Image-Pro Plus version 4.1.0.0 software program (Media Cybernetics, Bethesda, Maryland, USA). For each muscle section, the total number of muscle fibres and the number of immunofluorescence-positive muscle fibres were counted.

TABLE I
EXPERIMENTAL GROUPS

Group	Treatment	Drug dose (mg/kg/day)
A	Surgery + cycl A	2*
B	Surgery + cycl A	15†
C	Surgery + tacr	1*
D	Surgery + tacr	8†
E	None	–
F	Surgery	–

*Low dose. †immunosuppressive dose. Cycl = cyclosporin; tacr = tacrolimus; – = not applicable

Statistical analysis

All histomorphometric data are presented as mean ± standard deviations. Statistical analysis was performed using the GraphPad Instat 3.06 software program (GraphPad Software, San Diego, California, USA). Overall analysis of differences between the means for each group was performed using Kruskal–Wallis non-parametric one-way analysis of variance. Dunn's test was used to compare significant differences between groups. Statistical significance was set at a p value of less than 0.05.

Results and analysis

There was a significantly lower proportion of type 1 muscle fibres in group F (surgery alone; 7 per cent) than in group E (healthy group; 12.1 per cent; $p = 0.0303$). There were no significant differences between these two groups regarding types 2a and 2b fibres.

In the four groups receiving both surgery and drugs, there was no significant difference regarding the proportion of types 1 and 2a muscle fibres. There was a significantly lower proportion of type 2b fibres in group B (surgery plus immunosuppressive cyclosporin A; 25.8 per cent; $p = 0.0043$) and group D (surgery

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plus immunosuppressive tacrolimus; 23.9 per cent; $p = 0.0043$) compared with group F (surgery alone; 38.9 per cent) (Figures 1, 2 and 3). There was a significantly lower proportion of type 1 fibres in group C (surgery plus low-dose tacrolimus; 4.8 per cent; $p = 0.0043$) and group B (surgery plus immunosuppressive cyclosporin A; 6.4 per cent; $p = 0.0079$) compared with group E (healthy group; 12.1 per cent). There was no significant difference between groups E and any of the four surgery plus drug groups as regards types 2a and 2b fibres.

There was no significant difference in the proportion of types 1, 2a and 2b muscle fibres, comparing group E (healthy group) and group D (surgery plus immunosuppressive tacrolimus).

Discussion

Both denervation and reinnervation affect the intrinsic laryngeal muscles, and the correlation between these effects and muscle recovery remains controversial. In denervated skeletal muscle, a switch from type 1 to type 2a to type 2b muscle fibres is observed, which is reversible after reinnervation. In laryngeal muscles, DelGaudio and Sciote[11] found a decrease in anti-myosin heavy chain type 1 expression in denervated posterior cricoarytenoid muscle, in accordance with our results; this would indicate a switch from slow to fast muscle fibres. Kingham *et al.*[12] stated that modification of muscle fibre distribution is specific to the laryngeal muscle and to the animal model. They found increased type 1 fibre distribution and decreased types 2a and 2b fibre distribution after posterior cricoarytenoid denervation in pigs. Our results in rats showed a decreased proportion of type 1 fibres, without changes in types 2a and 2b fibres, indicating that the posterior cricoarytenoid muscle reacts to denervation like a skeletal muscle, with a switch from slow fibres to fast fibres.

Muscle fibres other than types 1, 2a and 2b have been reported. These 'other fibres' have mainly been identified as type 2x in the literature.[13] Some authors have reported that laryngeal reinnervation leads to a quick increase in type 2x fibres, and a decrease in types 2b and Eo fibres, via transitions in hybrid fibres co-expressing several myosin heavy chain types.[13,14] The proposed transition is from type 2b/Eo to type 2x/2b/Eo to type 2c/Eo to type 2x. The hypothesis involves a reinnervation of type 2b/Eo fibres by axons initially innervating type 2x fibres. There is no specific monoclonal antibody for myosin heavy chain type 2x in rats, so we could not study the expression of this fibre type, nor, therefore, type 2x fibre distribution. In our study, the observed decrease in the proportion of type 1 fibres without alteration in types 2a and 2b fibres was probably due to an increased proportion of type 2x fibres. The molecular mechanisms implicated in the regulation of the fast fibre subtypes remain poorly understood.

There are no previous reports regarding the influence of calcineurin inhibitors on laryngeal intrinsic muscle fibre distribution after reinnervation (assessed immunohistochemically). A positive effect of cyclosporin A on axonal survival after nerve grafting has been reported in earlier publications, but no functional improvement was demonstrated.[7] In skeletal muscles, several authors have demonstrated a neuroregenerative and functional improvement following tacrolimus administration after

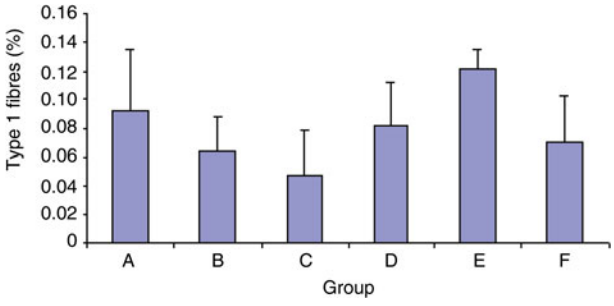

FIG. 1

Proportion of myosin heavy chain type 1 muscle fibres in the posterior cricoarytenoid muscle, on immunohistochemical analysis, 45 days after non-selective reinnervation by section-anastomosis of the vagus nerve. A = Surgery + low-dose cyclosporin A; B = surgery + immunosuppressive-dose cyclosporin A; C = surgery + low-dose tacrolimus; D = surgery plus immunosuppressive-dose tacrolimus; E = no treatment; F = surgery alone

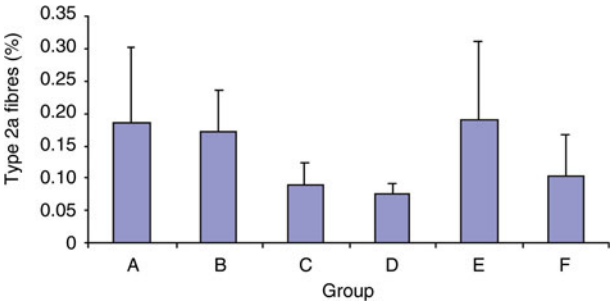

FIG. 2

Proportion of myosin heavy chain type 2a muscle fibres in the posterior cricoarytenoid muscle, on immunohistochemical analysis, 45 days after non-selective reinnervation by section-anastomosis of the vagus nerve. Groups as in Figure 1.

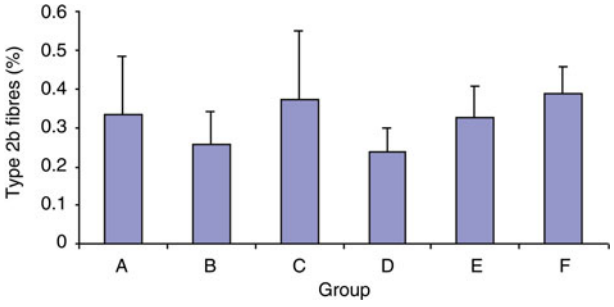

FIG. 3

Proportion of myosin heavy chain type 2b muscle fibres in the posterior cricoarytenoid muscle, on immunohistochemical analysis, 45 days after non-selective reinnervation by section-anastomosis of the vagus nerve. Groups as in Figure 1.

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nerve crush injury, nerve grafting, and reinnervation by nerve anastomosis.[15] This effect has not previously been studied in laryngeal muscles, despite their muscle specificities.

Our study found no significant differences in the proportion of types 1 and 2a muscle fibres, comparing the surgical groups. There were fewer type 2b fibres in the two immunosuppressive groups (group B, 50.5 per cent; and group D, 60.3 per cent) compared with the group receiving surgery alone (group F). This could mean either an increase in the proportion of hybrid type 2b/2x fibres, or an increase in the type 2b to 2x fibre switch. However, there was no significant difference in type 1 fibre distribution between the immunosuppressive tacrolimus group and the healthy group, whereas there was a significantly lower proportion of type 1 fibres in the immunosuppressive cyclosporin A group and surgery-alone group than in the healthy group.

- **The calcineurin inhibitor tacrolimus decreases slow fibre and increases fast fibre distribution in skeletal muscles**
- **It enhances peripheral nerve recovery, but laryngeal effects are unknown**
- **This study assessed effects of denervation and reinnervation of laryngeal muscle fibres**
- **Tacrolimus (at immunosuppressive dose) enhanced muscle fibre recovery after reinnervation**

Our main hypothesis is that, after reinnervation, the switch from slow muscle fibres to fast type 2b muscle fibres was less important in the immunosuppressive-dose tacrolimus group than in the surgery-alone group, and that cyclosporin A increased the switch to fast type 2x fibres. Therefore, immunosuppressive-dose tacrolimus enhanced the recovery of normal muscle fibre distribution, as a result of more effective reinnervation.

Conclusion

In the present study, 45 days after laryngeal reinnervation in rats, there was no difference in the distribution of types 1, 2a and 2b muscle fibres in the posterior cricoarytenoid muscle, comparing rats receiving an immunosuppressive dose of tacrolimus (after reinnervation) and rats receiving neither surgery nor drugs.

These results indicate that daily administration of tacrolimus enhances the recovery of normal laryngeal muscle fibres; hence, this drug may have a role in the transplantation setting.

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Address for correspondence:

Dr Philippe Gorphe,
Otorhinolaryngology and Head and Neck Surgery Department,
Rouen University Hospital,
76031 Rouen, France

E-mail: p_gorphe@yahoo.fr

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