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Long-term survival of an allografted submandibular gland in a miniature swine model given immunosuppressant drugs

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Abstract

We used a model of allografts of submandibular glands in miniature swine to test the requirements of immunosuppressants for the survival of such grafts. Animals in the first group ($n=6$) were given no immunosuppressant, and the submandibular glands were rejected within 7 days. Those in the low-dose group ($n=4$) were treated with a low dose of cyclosporine, methylprednisolone, and azathioprine. The allografts were rejected on days 15, 17, 19, and 20. The animals in the high-dose group ($n=6$) were treated with a high dose of cyclosporin, methylprednisolone, and azathioprine. Three allografts were rejected, on days 35, 48, and 60. One animal died from anaesthetic problems on day 30 after transplantation, and there were no signs of rejection in the allograft. The other two allografts survived for 100 days with secretory function and no signs of rejection. After we discontinued the immunosuppression on day 100, the two remaining allografts were rejected on days 121 and 128. Blood tests and biopsy specimens of the heart, lung, liver, and kidneys were normal in all animals that were given immunosuppressants. To our knowledge, this study is the first to describe long-term survival of allografted submandibular glands with secretory function in miniature swine.

Keywords: Submandibular gland; Homologous transplantation; Miniature swine

Introduction

Total xerophthalmia often results from exfoliative dermatitis, ocular pemphigus, trachoma, and congenital absence of the lacrimal glands.[1] Xerophthalmia is an important cause of blindness not only because the precorneal tear film is essential for corneal transparency but also because dry eyes develop keratitis with ulceration and opacification.[2]

In 1986, Murube-del-Castillo reported the transfer of an autologous submandibular gland as a way of treating severe xerophthalmia.[3] Since then, several groups have confirmed its value for this condition.[1,4–7] In brief, the submandibular gland is transferred to the temporal fossa, and end-to-end anastomoses are made between the external maxillary artery and the superficial temporal artery, and between the facial vein and the superficial temporal vein. The secretory duct is transplanted to the upper lateral conjunctival fornix so that glandular secretions drain to the eye, substituting for lacrimal fluid. Although clinical results have shown that the technique is effective for total xerophthalmia, there are some problems that it cannot overcome.

In some patients with xerophthalmia, the salivary gland fails to secrete enough fluid to keep the eye moist. For example, Binaghi et al.[8] reported that 11 of 26 patients (42%) who recovered from toxic epidermal necrolysis had dry eyes, and 7 of these 11 had reduced salivary flow. If a functionally compromised submandibular gland is transplanted in these patients, xerostomia will occur, and the transferred gland will not secrete sufficient fluid. Another problem in autotransplantation is that the submandibular gland would be lost entirely if the transplantation failed.

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Failure to correct insufficient secretion from the lacrimal gland will result in loss of eyesight. For patients whose submandibular glands are functionally compromised or missing, allotransplantation of a submandibular gland is an option. Miniature swine are similar to humans in anatomy, immunity, and histology of the submandibular gland, so valid information may be obtained from them for research into allotransplantation of submandibular glands.

Materials and methods

Experimental animals

The miniature swine (3–4 months old, weighing 10–20 kg) were provided by the Chinese Experimental Miniature Swine Stock Farm. The care procedures were in accordance with the Principles of Laboratory Animal Administration established by the Ministry of Science and Technology of the People's Republic of China. Two non-brood, weight-matched miniature swine were selected as donor and recipient, respectively, and one submandibular gland was transplanted between them. Male and female pigs were randomised. To avoid hyperacute rejection, blood was cross-matched before each transplant in each donor–recipient pair. A baseline laboratory test (complete blood count with differential, concentration of electrolytes, and liver function tests) was made in each animal before transplantation to assess the animal's general health.

Transplant model

The animals were fasted for 12 h and drank water freely before operation. Each animal was anaesthetised with an intramuscular injection of diazepam 10 mg and ketamine hydrochloride 30 mg/kg. Ketamine hydrochloride was injected once every 40–50 min during the procedure.

The vascularised allograft model that we used in this experiment was established in an earlier study. Briefly, the donor submandibular gland was orthotopically transplanted to the recipient and the duct was fixed to the oral vestibule. In the recipient, a submandibular incision was made and the gland removed. The proximal ends of the lingual artery and the external jugular vein on the same side were then prepared for anastomosis. In the donor, after a submandibular incision, the gland was dissected free but left on its maxillary arterial and external jugular venous pedicles. The duct was dissected free with a cuff of mucous membrane surrounding the orifice. The external maxillary artery and external jugular vein were then severed, and the donor submandibular gland was transferred to the recipient site and revascularised by anastomosing the artery of the gland to the lingual artery, and the vein of the gland to the external jugular vein using microvascular techniques. The secretory duct was drawn out through a subcutaneous tunnel and its end fixed to the oral vestibule.

Experimental groups and immunosuppressive protocol

The recipients of allotransplanted submandibular glands were assigned to one of three groups: the first group ($n=6$) received no immunosuppressants.

The low-dose group ($n=4$) received once-daily triple immunosuppressive drugs. The cyclosporin (Sandimmun injection, 50 mg/ml; Novartis Pharma AG, Switzerland) (15 mg/kg/day) was diluted (1:5) in normal saline and given intramuscularly, starting on the day before the operation and continuing for 7 days. From the seventh day cyclosporin was given by mouth (100 mg/ml; North China pharmaceutical Group Corporation, China) (30 mg/kg/day for 7 days and then 15 mg/kg/day as maintenance dose until the gland was rejected). Methylprednisolone (Solu-medrol; Pharmacia & Upjohn, Belgium) was given intramuscularly at a dose of 1 mg/kg/day starting on the day before operation until rejection occurred. Azathioprine (Imuran tablets, Glaxo Wellcome, UK) was given orally at a dose of 2 mg/kg/day starting on the day before operation until the gland was rejected.

The high-dose group ($n=6$) was given once-daily triple immunosuppressive drugs. The cyclosporin (15 mg/kg/day) was diluted (1:5) in normal saline and given intramuscularly startimg on the day before the operation and continuing for 7 days. From the seventh day after operation, it was given by mouth (45 mg/kg/day for 7 days and then 25 mg/kg/day as maintenance dose until the gland was rejected The same doses of methylprednisolone and azathioprine used in the low-dose group were also used in the high-dose group.

Doses of drugs were not adjusted depending on the clinical condition of the animals. In the high-dose group, the immunosuppressive agents were discontinued in the recipient animals in which the allograft survived at 100 days after transplantation, but the observations were continued.

Postoperative care

After transplantation, recipients were housed in separate cages and fed a liquid diet and water freely for 7 days. For prophylaxis against infection, penicillin G, 800,000 U/day were given intramuscularly for 7 days. For the first 7 days, the recipient was anaesthetised daily with 30 mg/kg ketamine hydrochloride and the surface of the duct orifice was flushed to avoid obstruction. In all animals, the general health, surgical incision, food intake, and bowel movements were observed daily.

Complete blood counts with differential, concentrations of electrolytes, and liver function tests were measured three times during the first week and then weekly thereafter. Trough concentrations of cyclosporin at 24 h were measured on days 30, 70, and 100 by fluorescence polarisation immunoassay (FPIA) in those animals in which the allograft had not been rejected.

Assessment of results

Results were assessed by visual inspection of secretions and histological examination of the transplanted submandibular gland. Secretions from the transplanted gland were inspected daily for the first 7 days, and then two to three times a week. The allograft was considered to be surviving when secretions were seen at the orifice of the duct. If secretion ceased (indicating lack of function), rejection was suspected. The recipient was then anaesthetised with ketamine hydrochloride 30 mg/kg and an open biopsy was taken, and a part of the allograft was removed for histological examination. At the same time, a complete blood count with differential, electrolyte concentrations, and liver function tests was done, and whole blood concentrations of troughs of cyclosporin were also measured by FPIA.

Histopathology

The specimens of submandibular gland were fixed in 10% formaldehyde, and sections were stained with haematoxylin and eosin to decide whether there were signs of rejection. If rejection of the allograft was confirmed by histological examination, the recipient was killed and a complete necropsy was done. Light and electron microscopic examinations were made on the allografted submandibular glands that survived to day 100.

Results

All operations were successful, and there were no complications. The operation time varied between 4 and 5 h. The mean warm ischaemic time was 1–3 min and the mean cold ischaemic time 30–40 min.

The recipient animals were generally in good health and physical condition. The control group ($n = 6$) were given no immunosuppressant, and the submandibular glands were rejected within 7 days. In the low-dose group, the four recipients were given a small dose of cyclosporin, and the allografts were rejected on days 15, 17, 19, and 20 after the operation. In the high-dose group ($n = 6$), the survival time of the allografts was considerably prolonged. Three allografts were rejected on days 35, 48, and 60, respectively. One recipient died of an anaesthesia-related incident on day 30 after transplantation and there were no signs of rejection in the allograft. The other two allografts survived for 100 days with no signs of rejection (Table 1). After the immunosuppression had been discontinued on day 100, the two remaining allografts were rejected on days 121 and 128, respectively. Another recipient in the high-dose group contracted pneumonia on day 42, and a blood test showed leucocytosis. After being given penicillin (800,000 U) intramuscularly once daily for 7 days, the animal showed no further sign of infection and the blood count was normal.

Histological examinations after transplant

All biopsy specimens taken after cessation of secretion of the allograft showed signs of rejection. The rejected gland felt hard and showed histological changes. In a normal submandibular gland, there are no inflammatory cells in the parenchyma of the gland (Fig. 1). Inflammatory cells had infiltrated in the parenchyma of the rejected glands. Most of these cells were lymphocytes and the rest were monocytes, plasmocytes, and eosinophilic granulocytes. There were multiple thrombotic and haemorrhagic foci in the parenchyma of the gland. At an advanced stage of rejection, inflammatory cells were scattered throughout the gland, and the acini were destroyed (Figs. 2 and 3).

Biopsy specimens were taken from the two miniature swine with the allografts that survived to day 100. Each gland was small, rosy, and soft. The blood inside was bright red and histological examination showed normal acinar structure. There were sporadic inflammatory cells in the interstitial tissue. The glandular lobule was slightly atrophic, the interstitial substance had increased, and the parenchyma had shrunk. The volume of both serous and mucous cells was reduced. Compared with a normal submandibular gland, the distribution of the allograft acini was irregular (Fig. 4). Under the electron microscope, the gland cells looked normal, and organelles and secretory granules were seen. The abundant secretory granules in the secretory cells suggested that the function of these cells had been maintained (Fig. 5).

All recipient miniature swine were killed when rejection was confirmed by histological examination. The necropsies showed no pathological changes in liver, heart, lung, or kidney.

Table 1
Whole blood concentration of cyclosporin in the high-dose group (μg/L)

No.	Concentration (day 30 after transplantation)	Concentration (day of rejection or discontinued medication)
1	208.56	107.55 (day 35, rejection)
2	312.54	312.54 (day 30, death from anaesthetic accident, no sign of rejection)
3	661.91	167.54 (day 48, rejection)
4	386.12	346.85 (day 60, rejection)
5[a]	375.84	356.97 (day 100, no sign of rejection)
6[b]	365.52	386.54 (day 100, no sign of rejection)

[a] 418.67 μg/L on day 70, no sign of rejection.
[b] 354.85 μg/L on day 70, no sign of rejection.

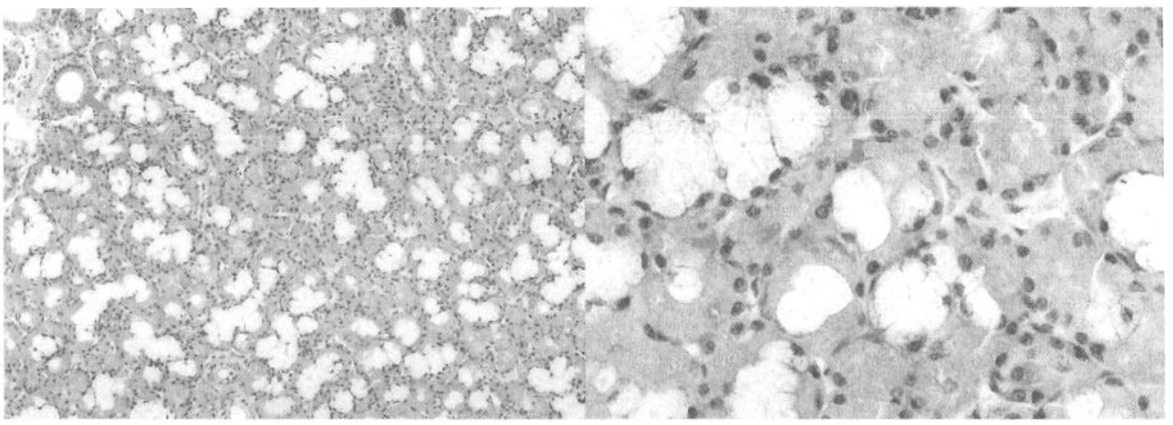

Fig. 1. Histological appearance of a normal submandibular gland. Serous cell (→); mucous cell (↓); duct (↑) (haematoxylin and eosin, original magnification: left, ×10; right, ×40).

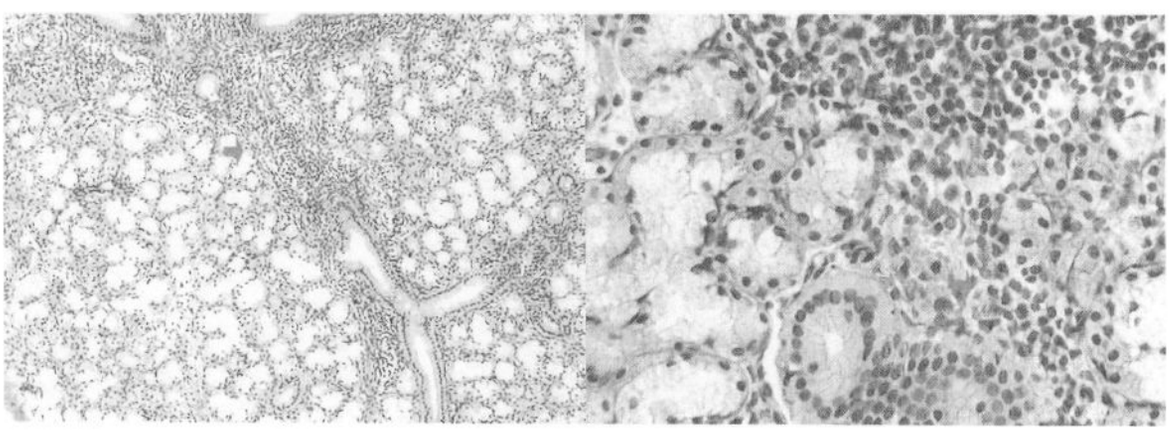

Fig. 2. Histological appearance of the rejected submandibular gland after transplantation in the control group. The structure of the acini is undisturbed, and inflammatory cells (→) can be seen between the lobules of the gland. Lymphocytes and plasmocytes surround the duct. No fibroblasts are seen (haematoxylin and eosin, original magnification: left, ×10; right, ×40).

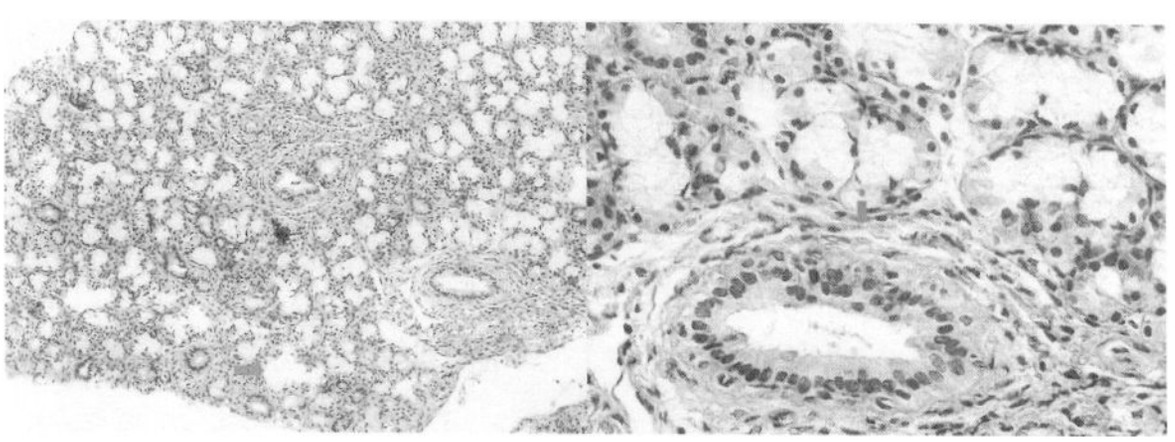

Fig. 3. Histological appearance of the rejected submandibular gland on day 35 after allotransplantation in the high-dose group. Lymphocytes and plasmocytes are dispersed in the parenchyma of the gland, and acini are damaged (→). There are fibroblasts (↓) around the ducts (haematoxylin and eosin, original magnification: left, ×10; right, ×40).

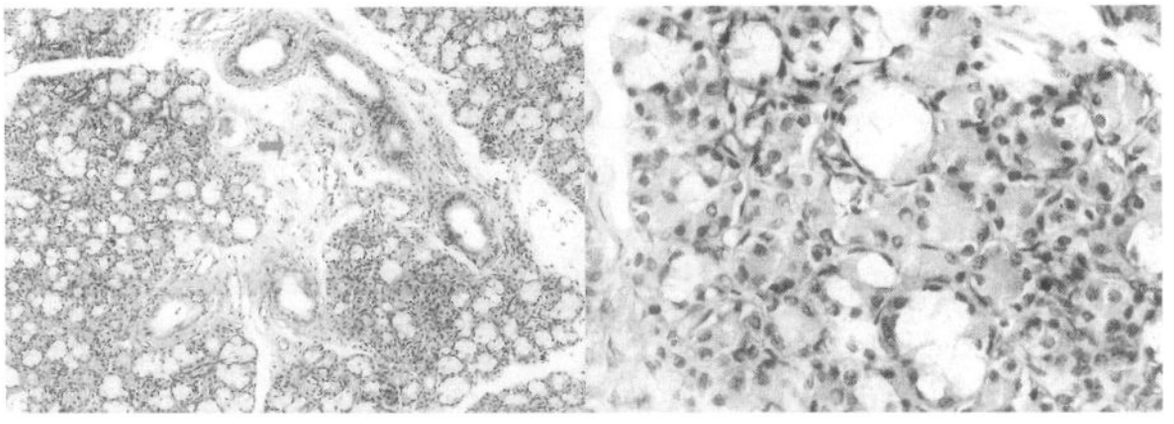

Fig. 4. Histological appearance of the living submandibular gland on day 100 after allotransplantation in the high-dose group. The structure of the acini appear normal, and there are sporadic lymphocytes (→) in the interstitial tissue. The disposition of the acini of the allograft is irregular and there are no signs of rejection (haematoxylin and eosin, original magnification: left, ×10; right, ×40).

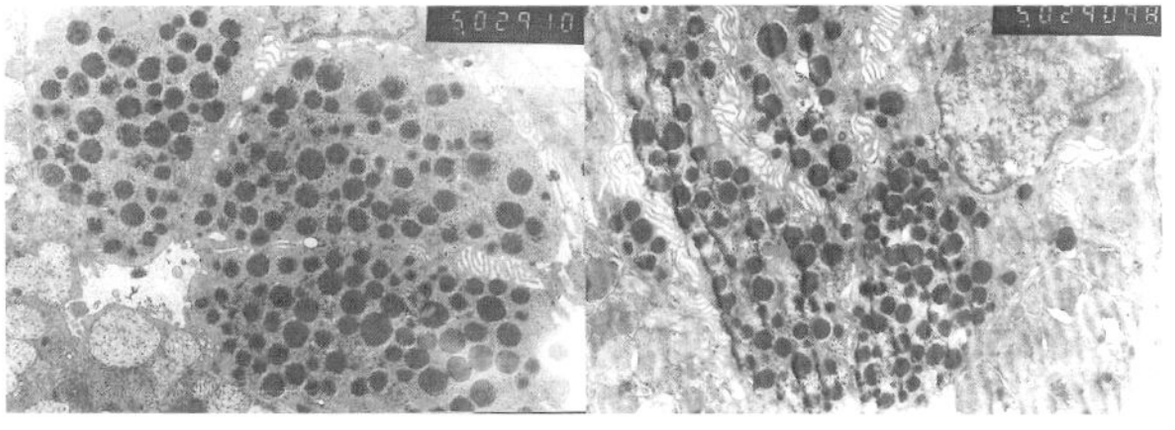

Fig. 5. Ultrastructure of the normal submandibular gland (left) and living submandibular gland on day 100 after allotransplantation in the high-dose group (right). The glandular cells of the living allograft look normal and digitations are clearly seen. There are abundant secretory granules (→) in the secretory cells (electron microscopic magnification ×5000).

Discussion

Recent allotransplantations of hand and larynx have opened a new era for improving quality of life with acceptable risks.[9,10] For those patients with total xerophthalmia who cannot be treated by autotransplantation of the submandibular gland, allotransplantation is a feasible alternative to prevent blindness. Previous experimental studies and clinical applications of autotransplantation of the submandibular gland have aided the understanding of the vascular anatomy of the submandibular gland, and surgeons have mastered the techniques for removal and implantation of this organ.[1–7] Allotransplantation of the submandibular gland is therefore more of an immunological than a surgical problem.

It is essential that the less toxic methods of controlling immunological rejection, toxicity, and the alteration of the submandibular gland be tested in appropriate animal models. In 1979, Faarup and Bing[11] made allotransplantations of the submandibular gland in mice and found no structural signs of rejection. However, in their study, the submandibular gland was not vascularised. In 2000, Wang et al.[12] published a model of revascularised allotransplantation of the submandibular gland in rabbits, to which no immunosuppressive drugs were given. Miniature swine are excellent for preclinical studies because of their immunological and anatomical similarities to humans, and they are extensively used in transplantation research.[13]

With an eye towards doing clinical transplants of submandibular glands, we have investigated the effectiveness and toxicity of immunosuppressants and seen histological changes in the submandibular gland after allotransplantation in miniature swine.

The introduction of cyclosporin in the early 1980s was a major breakthrough, and the drug was later used extensively in allotransplantation of organs and tissues. In this study, three immunosuppressive drugs (cyclosporin, methylprednisolone, and azathioprine) were given to find out whether the allografts of submandibular glands survived and main-

tained their secretory function without serious complications or side effects.

Histological examination showed no lesions in the recipients' liver, kidneys, heart, or lungs. Blood test results were also within the reference range. The doses of cyclosporin, methylprednisolone, and azathioprine delay rejection significantly without any major side effects in miniature swine. In the high-dose group, the two longest surviving allografts were rejected after immunosuppression had been discontinued. To achieve longer survival of the allograft, it is necessary for recipients to take immunosuppressive agents continuously.

Infection is thought to be one of the causes of failure of organ transplantation. In general, immunosuppressive drugs make the subject more susceptible to infection, and the swine allograft model's living conditions make it difficult to avoid infection after operation. In this study, the operations were done under aseptic conditions, and the pens were kept clean postoperatively. Antibiotics were given to prevent infection in the early period after operation. Except for one case in which pneumonia developed on day 42, no local or widespread infection occurred in the other recipients.

As methods of transplantation improve, rejection becomes the main cause of failure of a graft.

When there is precise tissue matching between donors and recipients, the results of transplantation are more successful and a lower dose of immunosuppressive agents or none at all may be effective. To the best of our knowledge, our study is the first to describe long-term survival of a submandibular gland allograft in a miniature swine model using immunosuppression. With a clinically acceptable risk, submandibular gland allotransplantation has the potential to treat total xerophthalmia and prevent blindness.

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