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Effects of Phenylephrine on Transplanted Submandibular Gland

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J Dent Res 85(12):1106-1111, 2006

ABSTRACT

Autotransplantation of the submandibular gland is a potential treatment for severe keratoconjunctivitis sicca. However, one of the major barriers to this procedure is that secretions from the transplanted gland decrease shortly after the operation, which may lead to obstruction of Wharton's duct, or even to transplantation failure. Using a rabbit model, we investigated whether phenylephrine could improve the secretion from the transplanted gland. We found that phenylephrine treatment significantly reversed the decrease in salivary secretion after transplantation, enhanced the expressions of α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor mRNA, and ameliorated atrophy of acinar cells. Furthermore, phenylephrine also induced translocation of aquaporin-5 from the cytoplasm to the apical membrane, and increased the levels of phospho-ERK1/2, ERK1/2, phospho-PKCζ, and PKCζ in the transplanted gland. These results indicate that phenylephrine treatment moderates structural injury and improves secretory function in the transplanted submandibular gland through promoting α_1-adrenoceptor expression and post-receptor signal transduction.

KEY WORDS: submandibular gland autotransplantation, α_1-adrenoceptor, phenylephrine.

INTRODUCTION

Keratoconjunctivitis sicca is a disease characterized by reduction or lack of tears, with serious complications. Despite the generally efficient pharmaceutical tear substitutes, tarsorrhaphy, or occlusion of the tear drainage system, individuals severely affected did not gain adequate relief of discomfort and often experienced corneal changes (Sieg *et al.*, 2000). Autotransplantation of the submandibular gland is one of the effective approaches for treating severe keratoconjunctivitis sicca (Murube-Del-Castillo, 1986; Geerling *et al.*, 1998; Sieg *et al.*, 2000; Yu *et al.*, 2004). However, almost all individuals experience a post-operative reduction of secretion of the transplanted glands from 5 days to 3 mos, which may lead to obstruction of Wharton's duct, or even to failure of the transplantation (Yu *et al.*, 2004). Therefore, if the success rate of transplantation is to be increased, it is critical that the secretion of transplanted glands be improved in the early post-operative stage.

It is well-known that the activation of cholinergic receptors and α-adrenoceptors is the main stimulus for fluid secretion, while β-adrenoceptor stimulation causes protein secretion in submandibular glands (Castle and Castle, 1996, Baum and Wellner, 1999). Since secretion of transplanted glands could be increased by physical activity, massage, and hot compresses instead of by food ingestion, we speculated that the increased secretion in the transplanted gland might be derived from adrenoceptor activation. Expressions of α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor mRNAs were detected in rat and rabbit submandibular glands (Piao *et al.*, 2000; Boekman *et al.*, 2004). Activation of α_1-adrenoceptor by sympathetic neurotransmitters has been found to evoke salivary fluid secretion in both innervated and denervated rat submandibular glands (Bylund *et al.*, 1982; Elverdin *et al.*, 1984; Baum and Wellner, 1999). Zhu *et al* utilized a rabbit model to examine the morphological changes in autotransplanted submandibular glands (Zhu *et al.*, 2002). They found that there were atrophic changes in secretory cells within 60 days after transplantation (Zhu *et al.*, 2001). However, the regulation of the secretion of denervated transplanted submandibular glands remains unclear. In this study, we investigated whether phenylephrine could affect the structure and function of the autotransplanted submandibular gland in rabbits.

MATERIALS & METHODS

Experimental Animals

Healthy male New Zealand white rabbits, weighing 2.4 ± 0.3 kg each, were used. All experimental procedures were in accordance with the Guidance of the Chinese Ministry of Public Health for the care and use of laboratory animals.

Received January 15, 2006; Last revision July 8, 2006; Accepted September 5, 2006

Reagents and Antibodies

Phenylephrine was purchased from Sigma (St. Louis, MO, USA). Antibodies to aquaporin-5 (AQP5), proliferating cell nuclear antigen (PCNA), protein kinase Cζ (PKCζ) and phospho-PKCζ, extracellular signal-regulated kinase (ERK1/2) and phospho-ERK1/2, and FITC-conjugated secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody to actin was purchased from Oncogene (Cambridge, MA, USA).

Experimental Groups and Operative Technique

With the rabbit under sodium pentobarbital (20 mg/kg body wt) anesthesia, its right submandibular gland, with the Wharton's duct and related blood vessels, was isolated from the submaxillary triangle. The freed gland was transplanted to the left temporal region and revascularized by anastomosis of the artery of the gland to the distal part of the external carotid artery, and the vein of the gland to the temporal vein. A polyethylene tube was inserted into the Wharton's duct and left outside the temporal skin. Rabbits were randomly divided into 3 groups: control (without transplantation), transplanted, and phenylephrine (transplantation with phenylephrine treatment). Phenylephrine (10^{-3} mol/L, 100 μL) or normal saline (100 μL) was slowly infused into the Wharton's duct from post-operative days 1-7, while the animals were conscious. On post-operative day 7, glands were removed under anesthesia.

Measurement of Salivary Secretion

Five min after phenylephrine or saline perfusion, salivary flow was measured for 5 min, on post-operative days 1-7, by the insertion of a piece of moist filter paper (35 mm × 5 mm) through the cannula inserted into the Wharton's duct. All collections were made between 9:30 a.m. and 10:30 a.m. from the resting and conscious animals. Saliva collected from post-operative days 1-7 was pooled for biochemical analysis of the concentrations of electrolytes and the activity of α-amylase.

Reverse-transcriptase Polymerase Chain-reaction (RT-PCR)

Total RNA was purified with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was prepared from 5 μg of total RNA with M-MLV reverse-transcriptase (Promega, Madison, WI, USA). The primers for α_1-adrenoceptor subtypes (Table) were designed and synthesized (AoKe Biotech, Beijing, PRC) based on the mRNA sequence of rabbit α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor (GenBank-U81982, AF156106, and U64032). Amplification of β-actin was performed for internal standardization. DEPC H_2O instead of RT product and human α_1-adrenoceptor subtype full-length cDNA were amplified as negative and positive controls, respectively. The band densities were quantitated by the use of a gel electrophoresis image system.

Confocal Microscopy

The translocation of AQP5 was identified by a previously described method (Ishikawa *et al.*, 2005) with modifications. Paraffin-embedded sections were immunostained by goat polyclonal anti-AQP5 antibody at a 1:100 dilution, incubated with FITC-conjugated anti-goat IgG at a 1:200 dilution, and examined by confocal laser scanning microscopy (Leica TCS 4D CLSM, Heidelberg, Germany).

Light Microscopic and Transmission Electron Microscopic Observations

The submandibular gland sections were stained with hematoxylin-eosin so that morphologic changes could be evaluated by light microscopy. Specimens were fixed in 2% paraformaldehyde-1.25% glutaraldehyde for evaluation by transmission electron microscopy (TEM). Ultrathin sections were stained with uranyl acetate and lead citrate, and observed with a TEM (H-7000 electron microscope; HITA-CHI, Tokyo, Japan).

Immunohistochemical Staining

The submandibular glands were immunostained with goat polyclonal antibody against PCNA (1:100), by routine immunohistochemistry. Negative controls were incubated with goat IgG replacing the primary antibody. The PCNA-positive cells were counted in 10 different fields in each section under 400× magnification.

Western Blot Analysis

Equal amounts of protein from different groups were separated on 12% SDS-PAGE, electrotransferred to polyvinylidene difluoride membrane, and probed with primary antibodies and horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Filters were re-probed with antibody against actin after being stripped to ensure equal loading of the lanes.

Statistical Analysis

Data are expressed as means ± SEM. Comparison of means was performed by one-way analysis of variance (ANOVA), followed by Bonferroni's tests. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Saliva Flow Rate and Composition in Submandibular Glands

To evaluate the salivary flow rate and composition of the transplanted glands in the early phase, we collected saliva directly from the cannula inserted into the transplanted glands. The saliva secreted from transplanted glands was increased on post-operative day 1, decreased on post-operative day 3, and hardly detectable on post-operative days 4-7 (Fig. 1A). To avoid the effects of phenylephrine *via* vein administration on the cardiovascular system, we administered it to the transplanted glands through the cannula. In the preliminary experiments, 10^{-3} mol/L of phenylephrine (100 μL) effectively stimulated salivary secretion without inducing vasoconstriction in submandibular glands, monitored by a Laser Doppler instrument. The blood

Table. Primers for Rabbit α_1-Adrenoceptor Subtype mRNA

	Upper Primer	Lower Primer	Fragment Size	Tm
α_{1A}-adrenoceptor	5'-CCT TCT CCC TGG TCA TCT CC-3'	5'-CAC CAT GTC CTT ATG CTG CC-3'	671 bp	58 C
α_{1B}-adrenoceptor	5'-AGG AGC CGG CAC CCA ATG ATG A-3'	5'-GGC ACT GGC ACC CGA GGA T-3'	549 bp	58 C
α_{1D}-adrenoceptor	5'-CTC CGT GCG CCT GCT CAA GT-3'	5'-GGG TAG ATG AGT GGG TTC AC-3'	210 bp	58 C
β-actin	5'-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG GCG-3'	5'-CGC CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'	843 bp	58 C

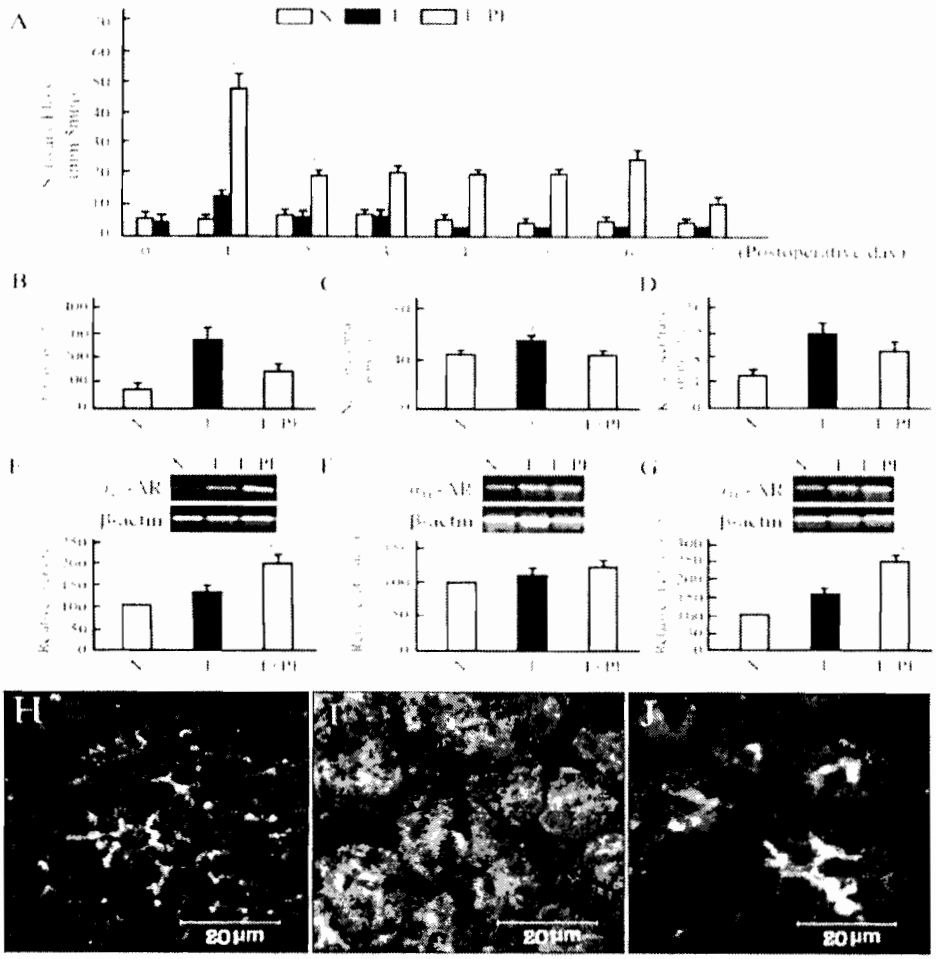

Figure 1. Effects of phenylephrine on salivary secretion, mRNA expressions of α_1-adrenoceptor subtypes, and localization of AQP5 in transplanted glands. Salivary flow **(A)**, activity of α-amylase **(B)**, sodium concentration **(C)**, and potassium concentration **(D)** of the saliva were measured as described in MATERIALS & METHODS. The transplanted glands were administrated phenylephrine from post-operative day 1. The expressions of α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor mRNA were detected by RT-PCR in control, transplanted, and phenylephrine-treated glands (n = 6) **(E,F,G)**. All values are given as means ± SEM. N, control submandibular gland; T, transplanted gland; T + PE, transplanted gland with phenylephrine treatment. $^*P < 0.05$ and $^{**}P < 0.01$ compared with N; $^{\#}P < 0.05$ compared with T. The reactivity to anti-AQP5 antibody was detected with FITC-conjugated secondary antibody. Sections were examined by confocal microscopy. **(H)** Control submandibular gland. AQP5 was located in both the apical membrane and the cytoplasm. **(I)** Transplanted gland. AQP5 was diffused in cytoplasm. **(J)** Translocation of AQP5 from cytoplasm to the apical membrane in phenylephrine-treated gland. Bars (H,I,J): 20 μm.

pressure and heart rate of rabbits were unchanged under this dosage (data not shown). Treatment with phenylephrine markedly increased the secretion from transplanted glands from post-operative days 1-7. The α-amylase activity and concentrations of sodium and potassium in saliva collected from transplanted glands were higher than those in the control group. After phenylephrine treatment, the concentration of sodium was significantly decreased to control levels, while the α-amylase activity and the concentration of potassium remained higher compared with the controls (Figs. 1B, 1C, 1D).

Expression of α_1-Adrenoceptor Subtype mRNA in the Submandibular Gland

The PCR products were visible in the gel at the expected sizes of 671 bp, 549 bp, and 210 bp for α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor subtypes, respectively (data not shown). The expressions of α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptor mRNA in transplanted glands were increased by 29.54%, 9.59%, and 45.61%, respectively, compared with those in the control, and were further increased by 78.54%, 22.67%, and 125.78%, respectively, after phenylephrine treatment (Figs. 1E, 1F, 1G).

Effect of Phenylephrine on AQP5 Translocation

AQP5 was localized in both the apical membrane and the cytoplasm under unstimulated conditions (Fig. 1H). AQP5 was dispersed in the cytoplasm in transplanted glands on post-operative day 7 (Fig. 1I). After phenylephrine treatment, AQP5 was translocated from the cytoplasm to the apical membrane (Fig. 1J).

Histologic and Ultrastructural Changes in Transplanted Glands

Normal acinar and ductal cells in control submandibular glands were displayed by light microscopy (Fig. 2A). Acinar cells in transplanted glands were shown to atrophy, compared with controls, on post-operative day 7 (Fig. 2B). After phenylephrine treatment for 7 days, the glandular atrophy was ameliorated, and its morphologic manifestation was much closer to that of the control (Fig. 2C). The acinar cells in control submandibular glands contained many low-matrix-density secretory granules in the cytoplasm, under TEM (Fig. 2D), but there were fewer in transplanted glands on post-operative day 7 compared with the controls (Fig. 2E). In the phenylephrine-treated glands, abundant low-density secretory granules were observed in acinar cells, and their sizes were similar to those seen in the cells in the control glands (Fig. 2F).

PCNA-positive Cells in Submandibular Glands

A few brown nuclear PCNA-positive cells were identified in ductal cells in the control submandibular glands (Fig. 3A). PCNA-positive cells were increased in both acinar and ductal cells in the transplanted glands (Fig. 3B), and were further increased in phenylephrine-treated glands (Fig. 3C). The numbers of PCNA-positive cells in control, transplanted, and phenylephrine-treated glands were 4.9 ± 2.39, 12.4 ± 3.85, and

94.1 ± 10.01 *per* high-power field, respectively (Fig. 3D).

Effects of PE on PKCζ and ERK1/2 Protein Expression in Submandibular Glands

To determine the signal molecular expression related to the proliferation in transplanted glands, we detected PKCζ and ERK1/2 by Western blot. The results showed that the expressions of phospho-PKCζ, PKCζ, phospho-ERK1/2, and ERK1/2 were elevated in transplanted glands and further increased in phenylephrine-treated glands compared with the control glands (Figs. 3E, 3F).

DISCUSSION

Earlier studies have reported that parasympathetic and sympathetic denervation of the rabbit submandibular gland caused atrophy of the acinar cells (Kyriacou and Garrett, 1998). In this study, we demonstrated that the atrophy of the acinar cells, in addition to a decrease in secretion, is an early morphological change in transplanted glands. Phenylephrine treatment could improve secretion and ameliorate the atrophy of the transplanted glands in the early phase after transplantation. It is well-known that denervation induces "super-sensitivity secretion" and "degeneration secretion" in salivary glands, due to the release of neurotransmitters from the degenerating nerves during the early stages after denervation (Delfs and Emmelin, 1974; for review, see Proctor, 1999). Consistent with these early observations, our clinical studies also found that salivary flow was increased soon post-operatively and decreased on post-operative day 5 in transplanted glands (Yu *et al.*, 2004). Our results indicate that the administration of phenylephrine might aid in the prevention of ductal obstruction after transplantation by stimulating secretion.

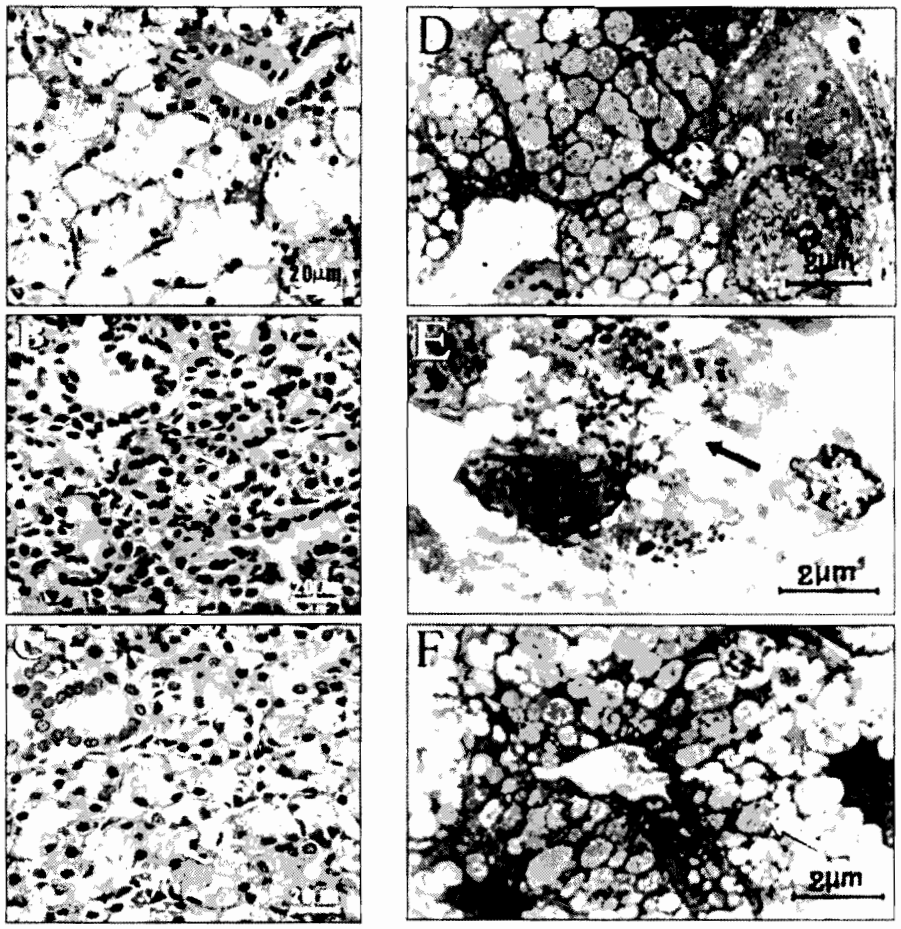

Figure 2. The histologic structure and ultrastructure of transplanted submandibular glands. All submandibular gland tissues were removed on post-operative day 7. **(A)** Histologic structure of a control submandibular gland. An acinar cell is indicated by an arrow. **(B)** Histologic structure of transplanted gland. Acinar cells show size reduction (arrow). **(C)** Histologic structure of phenylephrine-treated gland. Acinar cells show only mild size reduction (arrow). Light microscopy, magnification 400x. Bars (A,B,C): 20 μm. **(D)** Ultrastructure of control submandibular gland. Low-matrix-density secretory granule in acinar cell is indicated by an arrow. **(E)** Ultrastructure of transplanted submandibular gland. The low-matrix-density secretory granules (arrow) were decreased as compared with those in the control submandibular gland. **(F)** Ultrastructure of phenylephrine-treated gland. The low-density secretory granules (arrow) are similar to those in the control submandibular gland. TEM magnification, 5000x. Bars (D,E,F): 2 μm.

In agreement with our previous clinical observations in human submandibular gland transplantation (Li *et al.*, 2002), we found that the K^+ concentration was significantly increased, while the Na^+ concentration and α-amylase activity were slightly increased in transplanted rabbit submandibular glands. It has been well-established that the primary saliva secreted from acini is a plasma-like fluid, with high concentrations of Na^+ and low levels of K^+. When saliva flows through the duct system, its composition is greatly modified by ductal cells through re-absorption of Na^+ and Cl^- and release of K^+ and HCO_3^-. Stimulation of adrenergic or cholinergic receptors activates these ion transporters in ductal cells. The increase in K^+ concentration in the saliva from transplanted glands, as observed in our studies, indicates that the ductal cells in these glands were activated by transplantation. The amylase increase was likely due to up-regulated acinar secretion of the protein in transplanted glands. Interestingly, phenylephrine did not further increase K^+ levels. These observations may result from the activation of the cells in transplanted glands, due to the "degeneration secretion" of the neurotransmitters in the early stage after transplantation. Further stimulation of the cells with phenylephrine failed to trigger any additional response. The accurate underlying mechanisms mediating these changes are currently under further investigation.

Little is known about alterations in α_1-adrenoceptor subtypes in the transplanted organs. We found that mRNAs of 3 subtypes of α_1-adrenoceptors, especially α_{1A}- and α_{1D}-adrenoceptors, were elevated in transplanted glands and further

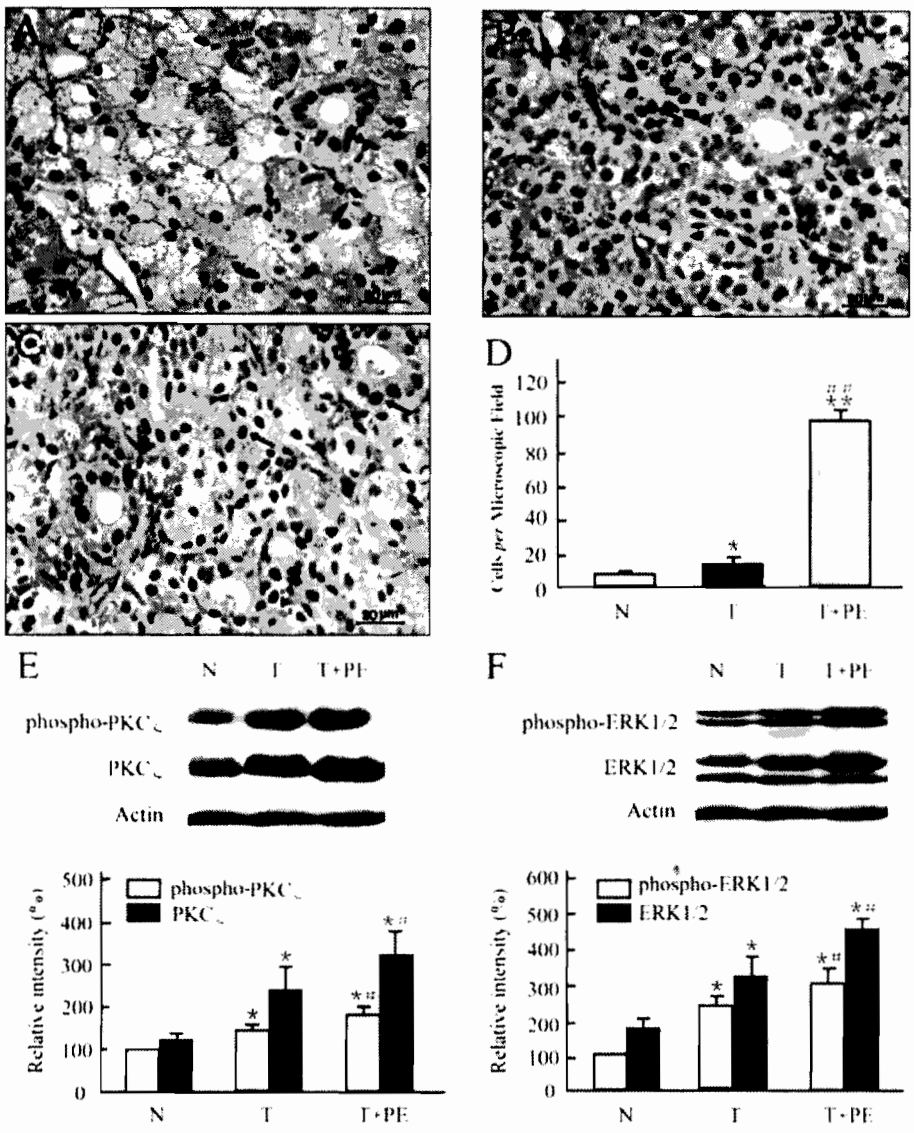

Figure 3. Alterations of PCNA and MAPK signal pathways in transplanted submandibular glands. Immunohistochemical localizations of PCNA in control submandibular gland **(A)**, transplanted gland **(B)**, and phenylephrine-treated gland **(C)** are shown. PCNA-positive cell is indicated by an arrow. Light microscopy, magnification 400x. Bars (A,B,C): 20 μm. **(D)** Semiquantitative scoring of PCNA-positive cells (n = 6). The PCNA-positive cells were counted in 10 different fields in each section (1 section/each animal) under 400x magnification. **(E)** Western blot analysis of phospho-PKCζ and PKCζ. An 80-μg quantity of protein was separated by 12% SDS-polyacrylamide gel electrophoresis and immunoblotted either with an antibody specific for phospho-PKCζ or for total PKCζ. **(F)** Western blot analysis of phospho-ERK1/2 and ERK1/2. An 80-μg quantity of protein was separated by 12% SDS-polyacrylamide gel electrophoresis and immunoblotted either with an antibody specific for phospho-ERK1/2 or for total ERK1/2. Filters were re-probed with antibody against actin to ensure equal loading of the lanes. The blot is a representative of 5 separated animals in each group, with similar results. All values are given as means ± SEM. N, control submandibular gland; T, transplanted gland; T + PE, transplanted gland with phenylephrine treatment. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with N; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with T.

increased in phenylephrine-treated glands. Therefore, transcriptional induction of α_1-adrenoceptors could be a potential mechanism for sustained secretion, a chronic response to catecholamine in transplanted glands.

It is known that the activation of α_1-adrenoceptors plays an important role in regulating cell growth and proliferation through mitogen-activated protein kinase (MAPK) pathways (Alexandrov *et al.*, 1998; Lazou *et al.*, 1998). Enhanced cell proliferation after ischemia is considered a sign of initiation of regeneration of the gland tissue (Sieg *et al.*, 2000). In the present study, PCNA-positive cells were identified in transplanted glands and markedly increased in phenylephrine-treated glands. This suggests that the regenerative capacity of transplanted glands could be enhanced by phenylephrine. α_1-Adrenoceptor can activate MAPK through the G_q PKC Raf-1-dependent pathway (Post and Brown, 1996). PKCζ, an atypical PKC, has been implicated in the suppression of apoptosis, is required for ERK activation, and mediates the proliferation of a plethora of cell types (for review, see Hennige *et al.*, 2002; Gutcher *et al.*, 2003; Muscella *et al.*, 2003; Parmentier *et al.*, 2003). Importantly, we found that phenylephrine significantly increased the levels of phosphorylated PKCζ and ERK1/2 in transplanted glands. These results indicate that the activation of α_1-adrenoceptor by phenylephrine can promote the regeneration of transplanted glands by activating the MAPK signal pathway.

Moreover, localization of AQP5 may be critical to its function in salivary fluid secretion (for review, see Melvin *et al.*, 2005). Interestingly, our studies demonstrated that the activation of the α_1-adrenoceptor by phenylephrine could induce translocation of AQP5 from the cytoplasm to the apical membrane. The results suggest that the increased salivary secretion stimulated by phenylephrine may be mediated by the translocation of AQP5 in transplanted glands. In support of our observation, phenylephrine was found to induce the trafficking of AQP5 to the apical membrane in rat parotid cells (Ishikawa *et al.*, 1999).

In summary, our studies demonstrated that phenylephrine stimulated salivary secretion, α_1-adrenoceptor subtype mRNA up-regulation, and AQP5 translocation in transplanted glands. Phenylephrine also ameliorated atrophy and may promote cell proliferation in transplanted glands by activating PKCζ and

ERK1 2. Our results suggest that phenylephrine has the potential to be used for clinical prevention of the reduction of secretion of submandibular glands during the early phase of transplantation.

ACKNOWLEDGMENTS

This study was supported by grants from the National Nature Science Foundation of China (Nos. 30171016 and 30371545), the Ministry of Education (No. 104002), the Ministry of Science and Technology (No. 2003CCC00800), and the Program for Changjiang Scholars and Innovative Research Team in University. The authors thank Professor Cun-yu Wang at the University of Michigan School of Dentistry and Medicine, and Dr. Guo H. Zhang at the National Center for Research Resources, National Institutes of Health, USA, for reviewing the manuscript.

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