Antisense Oligodeoxyribonucleotide Targeting cAMP Response Element-Binding Protein Inhibits Growth of Rat Submandibular Gland *In Vitro*

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The transcription factor, cyclic AMP response element-binding protein (CREB), occurs in the nuclei of acinar cells of the rat submandibular gland during the early postnatal periods. To elucidate the role of CREB in the growth of submandibular gland, the effect of an antisense oligodeoxyribonucleotide (ODN) targeting CREB was examined in the organ culture system of neonatal rat submandibular gland. In the presence of antisense-CREB ODN in the culture media for 48 hr, a significant decrease in the growth of cultured gland was observed in terms of both the area of acinar epithelial tissue and ^3H -thymidine-labeling index of acinar cells, with concomitant decrease in

the nuclear immunoreactivity for CREB without any degenerative changes in the histology of the gland. In contrast, sense-CREB ODN exhibited no effect on acinar growth. Administration of the β -adrenergic agonist isoproterenol (IPR) induced enhanced proliferation of acinar cells, which was also inhibited by antisense-CREB ODN. In contrast, the proliferation of connective tissue cells was neither enhanced by IPR nor inhibited by antisense-CREB ODN. These results suggest that the transcription factor CREB plays specific roles in the proliferation of submandibular gland acinar cells during the early postnatal periods.

Key words: CREB, Antisense, Cell growth, Organ culture, Submandibular gland, Rat

I. Introduction

In the rodent submandibular glands, extensive cell proliferation and differentiation take place during the postnatal periods under the control of neuronal and hormonal factors [18, 21]. In particular, the first few postnatal weeks are dominated by proliferation of acinar epithelial cells, which is considered to be controlled primarily by the β -adrenergic mechanisms based on the following lines of evidence. 1) Coupling of sympathetic nerves and the β -adrenergic receptor/adenyl cyclase system is established in the gland during the first week [10]. 2) Surgical and chemical sympathectomy in the early postnatal periods causes a retardation in the gland development [36]. 3) Proliferation and hypertrophy of acinar cells are induced by administration of the β -adrenergic agonist isoproterenol (IPR) [9].

IPR is known to exert its action through binding to cell surface β -adrenergic receptor and activating the cyclic AMP (cAMP)/protein kinase A (PKA) signaling pathway [7]. In the rodent parotid and submandibular glands, IPR

causes salivary protein secretion (immediate effect) [34, 37] as well as proliferation (acute effect) [4, 6] and hypertrophy (chronic effect) [23, 30] of acinar cells. IPR-induced salivary protein secretion can reasonably be ascribed to an increase in the intracellular levels of cAMP which, through the PKA pathway, causes the activation by phosphorylation of some substrates associated with the exocytosis of secretory granule contents. A recent study pointed to vesicle-associated membrane protein 2 as a strong protein candidate that may be activated by PKA and function in exocytosis in rat parotid gland acinar cells [12]. In contrast, the intracellular mechanisms by which the signal of IPR leads to acinar cell proliferation and/or hypertrophy are not fully understood.

Cyclic AMP response element-binding protein (CREB) is a 43 kDa polypeptide that binds to a specific 8-nucleotide sequence of DNA known as cAMP-response element (CRE) [28, 31]. Upon elevation of intracellular cAMP, CREB is phosphorylated on the serine 133 residue by PKA and functions as transcription factor by binding to CRE and recruiting CREB-binding protein, an essential cofactor that interacts with the basic transcription machinery [15, 39]. In our previous study in the rat submandibular gland, we demonstrated the specific localization of CREB immunoreactivity in the nuclei of acinar

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cells during the first few postnatal weeks. Furthermore, the number of CREB-positive acinar cells showed transient increase after administration of IPR, suggesting that CREB plays important roles in acinar cell proliferation [1].

The present study was designed to further clarify the involvement of CREB by applying the deprivation of CREB with an antisense oligodeoxyribonucleotide (ODN) targeting CREB to the organ culture system of neonatal rat submandibular gland.

II. Materials and Methods

Preparation of ODNs

Synthetic 15-bp ODNs with the following sequences were designed according to the published rat CREB cDNA sequence [16] (GenBank accession number, X14788) and purchased from Japan BioService Co. (Asagiri, Japan) for use in the antisense inhibition experiments: antisense-CREB ODN, 5'TTGTCTGCTCCAGAG 3'; sense-CREB ODN, 5'CTCTGGAGCAGACAA 3', wherein the underlined bases were phosphorothioated. This antisense-CREB ODN was complementary to the sequence encompassing 11~25 nucleotides downstream of the translation initiation site. Homology search on DNA Data Bank of Japan using BLAST program indicated that this sequence has no homology at the score 24 bits or more with any other rat cDNA that may be expressed in the submandibular gland.

Organ culture and antisense inhibition

Wistar rats at 0 day after birth were anesthetized and the submandibular glands were dissected out. They were immediately immersed in cold Hanks' balanced salt solution (Nissui, Tokyo, Japan) and cut into 1-mm-cube blocks under the dissection microscope. The organ culture was performed according to the method described previously [2]. Each block was placed on a Millipore filter (Millipore, Bedford, MA), and 5~6 blocks were explanted onto a stainless steel mesh placed in an Falcon organ culture dish (Becton Dickinson, Franklin Lakes, NJ) that was filled with 1 ml of BJGb medium (Life Technologies, Rockville, MD) freshly supplemented with 0.5% ascorbic acid, 100 U penicillin/streptomycin, 10% fetal calf serum (Filtron, Brooklyn, Australia) and adjusted to pH 7.5. To examine the effect of deprivation of CREB, antisense- and sense-CREB ODNs were added at 25 μ M in the culture media of some explants from the beginning of the culture. Also, to examine the effect of β -adrenergic stimulation, *dl*-isoproterenol hydrochloride (IPR) (Wako Pure Chemicals, Osaka, Japan) was added at 0.2 μ g/ml to the media. The explants in dishes were cultured in a humidified, 95% air and 5% CO₂ atmosphere at 37°C for 48 hr. Some explants were pulse-labeled with 20 μ Ci/ml of [methyl-³H] thymidine (740 GBq/mmol, NEN, Boston, MA) for the last 1 hr of the culture period. Thereafter, the explants were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.2) for 4 hr at 4°C and processed for the mor-

phological analyses consisting of conventional histology, immunohistochemistry, and microautoradiography.

Immunohistochemistry

The fixed explants were rinsed in 30% sucrose/0.1 M phosphate buffer overnight at 4°C, frozen and cut into 10 μ m-thick sections on a cryostat. After drying, the sections were treated successively with 0.3% Triton X-100 in phosphate buffered saline (PBS) for 60 min, 0.3% oxygen peroxide in methanol for 10 min and 5% normal porcine serum in PBS for 30 min. Thereafter, the sections were incubated with rabbit anti-rat CREB antiserum (Upstate Biotechnology, Inc., Lake Placid, NY) at 1 : 2000 dilution overnight at room temperature. The specificity of this antibody had been confirmed in previous studies by ourselves and others [1, 14]. After extensive washing with PBS, the site of immunoreaction was visualized by incubating the sections successively with biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA), horse radish peroxidase-conjugated streptavidin (Dakopatts, Glostrup, Denmark), and with 0.01% 3,3'-diaminobenzidine tetrahydrochloride in the presence of 0.002% hydrogen peroxide in 50 mM Tris-HCl (pH 7.5).

Conventional histology and morphometry

The fixed explants were dehydrated in graded ethanol series, embedded in paraffin, cut into 5- μ m serial sections and mounted on glass slides. The sections were then stained with hematoxylin-eosin and subjected to observation with an Olympus BX50 microscope connected to a CCD video camera (Olympus/Victor KY-F55MD). At the 480-fold magnification, 6 rectangular fields 480000 μ m² in area and containing no interlobular connective tissues were selected randomly from different sections obtained from 2 explants. The relative area of acini to the total area was calculated for each field using public-domain NIH image software and was expressed as mean $\pm$ SD of 6 fields. The statistical difference between two values was examined by Student's *t* test and the difference with *p* < 0.01 was considered significant.

Detection of proliferating cells

The microautoradiography was performed as described previously [2]. Briefly, paraffin sections were prepared from the ³H-thymidine-labeled explants, dipped in Kodak NTB2 nuclear-track emulsion diluted with 0.33 M ammonium acetate, and were placed in a dark box at 4°C. After a 7-day exposure, the sections were developed and stained with hematoxylin-eosin and subjected to observation with the microscope/CCD video camera system. At the 40-fold magnification, 6 rectangular fields 120000 μ m² in area and containing no interlobular connective tissues were selected randomly from different sections obtained from 2 explants. The percent number of labeled cells (labeling index) was calculated separately for acinar and connective tissue cell populations in each field and was statistically analyzed in the same way as in the

Fig. 1. Effects of antisense-CREB ODN and IPR on the growth of cultured submandibular gland. (a–c) Photomicrographs showing the explants of neonatal submandibular gland at the beginning of culture (a) and after a 48 hr culture in the absence (b) and presence (c) of antisense-CREB ODN. $\times 300$. Note the difference in the relative areas of acinar epithelium and connective tissue. (d) Morphometry of the relative area of acinar epithelium in the explants before (P0+0) and after (P0+2) the culture with or without IPR and with antisense- or sense-CREB ODN. The values are significantly larger than the control before culture (*), larger than the control cultured without IPR (**), smaller than the control cultured without IPR (***) and smaller than the control cultured with IPR (****), at $p < 0.005$, $n = 6$.

morphometry.

III. Results

At the beginning of the culture, the rat submandibular gland at birth was composed of clearly demarcated acinar epithelial tissue interspersed among abundant loose connective tissue. The striated ducts were directly connected to the intercalated ducts, which branched off to form the acinar rudiments known as the terminal tubules (Fig. 1a). After a 48-hr culture, the overall size of the explant showed no apparent changes, whereas the area of acinar epithelial tissue expanded substantially due to the proliferation of primitive acinar cells (Fig. 1b). Although the cells of connective tissue also increased in number, the area of connective tissue showed no increase because of reduction in the area of intercellular space. As a result,

the relative area of acinar epithelium in the gland exhibited a significant increase from 7% at the beginning to 25% at the end of the culture period ($p < 0.005$) (Fig. 1b, d). In the gland cultured with antisense-CREB ODN, the proliferation of acinar cells was markedly inhibited, resulting in a significant reduction in the relative area of acinar epithelial cells to about one-third that of the control gland ($p < 0.005$) (Fig. 1c, d). In contrast, the increase in the number of connective tissue cells appeared to be unaffected. No degenerative changes were recognized in any portion of the gland cultured with antisense-CREB ODN. Administration of IPR produced about 2.5-fold increase in the relative area of acinar epithelium (Fig. 1d). In the presence of antisense-CREB ODN together with IPR, the relative area of acinar epithelium became significantly lower than that in the presence of IPR alone ($p < 0.005$) and was comparable to the level of the control gland. In

Fig. 2. Immunohistochemical detection of CREB in the explants of neonatal submandibular gland cultured for 48 hr in the absence of ODN (a) and in the presence of antisense-CREB ODN (b) or sense-CREB ODN (c). Note that the nuclear immunoreactivity in acinar epithelial cells in (a) is suppressed almost completely in (b) but is unaffected in (c). $\times 250$.

contrast, sense-CREB ODN caused no significant inhibition of the acinar growth of control gland cultured in the presence and absence of IPR.

To confirm the effectiveness of antisense-CREB ODN in depriving CREB protein, explants at the end of the culture period were examined with immunohistochemistry for CREB. In the control gland, the majority of acinar epithelial cells and connective tissue cells exhibited intense immunoreactivity in the nucleus (Fig. 2a). The presence of antisense-CREB ODN in the culture medium caused a substantial reduction in the intensity of CREB immunoreactivity in all cells, whereas sense-CREB had no effect (Fig. 2b, c).

Inhibition of acinar epithelial growth by antisense-CREB ODN was also evaluated in terms of uptake of ^3H -thymidine by proliferating cells. In the control gland, the labeling index was approximately 6% for acinar cells and 4.5% for connective tissue cells (Fig. 3a, e). The presence of antisense-CREB ODN in the culture medium caused a remarkable reduction in the labeling index of acinar cells to 2% ($p < 0.005$), whereas it had no significant effect on the labeling index of connective tissue cells (Fig. 2b, e). Administration of IPR produced about 2-fold increase in the labeling index of acinar cells ($p < 0.005$), whereas it had no significant effect on the labeling index of connective tissue cells (Fig. 3c, e). In the presence of antisense-CREB ODN together with IPR, the labeling index of acinar cells became significantly lower than that in the presence of IPR alone ($p < 0.005$) and was comparable to the level of control gland (Fig. 3d, e). Sense-CREB ODN caused no significant effect on the labeling indexes of acinar and connective tissue cells of control glands cultured in the presence and absence of IPR (Fig. 3e).

IV. Discussion

The present system of the organ culture of newborn rat submandibular gland for 48 hr has proved to reproduce many of the features of *in vivo* submandibular gland during the early postnatal periods, including the normal

acinar and duct structure, increase in the relative area of epithelial components to connective tissue components, proliferation of acinar epithelial cells, and responsiveness to growth-promoting activity of IPR [18, 21]. Using this system, the present study has clearly demonstrated that the antisense ODN targeting CREB causes a marked inhibition of the growth of submandibular gland. The antisense ODNs are widely used for blocking the expression of specific genes based on their ability to bind to the complementary mRNAs and prevent translation [35, 38]. The present growth inhibition by antisense-CREB ODN should be ascribed to decreased intracellular CREB levels, because 1) antisense-CREB ODN causes a marked reduction in the intensity of nuclear CREB immunoreactivity, and 2) sense-CREB ODN is incapable of the growth inhibition. Consequently, it is strongly suggested that CREB is involved in the mechanisms underlying the growth of rat submandibular gland during the early postnatal periods.

The present results have shown that the growth of cultured neonatal rat submandibular gland is primarily due to the proliferation of acinar epithelial cells, which occurs spontaneously in the culture medium supplemented with 10% fetal calf serum and is enhanced by administration of IPR. Furthermore, both of the spontaneous and IPR-induced proliferation of acinar cells are specifically inhibited by antisense-CREB ODN. Considering that IPR activates the PKA pathway through its binding to β -adrenergic receptor and subsequent increase of cytoplasmic cAMP levels [7], it seems reasonable to assume that IPR phosphorylates nuclear CREB in acinar cells and thereby activates expression of some cAMP-inducible genes responsible for cell proliferation. However, to our knowledge the present study is the first to demonstrate directly the involvement of CREB in IPR-induced proliferation of acinar epithelial cells. The present results also indicate that the proliferation of connective tissue cells is neither enhanced by IPR nor inhibited by antisense-CREB ODN, suggesting that CREB-dependent proliferation is unique to acinar cells in the submandibular

Fig. 3. Effects of antisense-CREB ODN and IPR on cell proliferation in the cultured submandibular gland. (a–d) Autoradiographs showing the explants of neonatal submandibular gland cultured for 48 hr in the absence (a, b) or presence (c, d) of IPR and in the absence (a, c) or presence (b, d) of antisense-CREB ODN, and pulse-labeled with ^3H -thymidine at the end of culture. Note the difference in the number of labeled cells with silver grains. $\times 250$. (e) The labeling index of acinar epithelial cells and connective tissue cells in the explants cultured with or without IPR and with antisense- or sense-CREB ODN. In acinar cells, the values are significantly larger than the control without IPR (*), smaller than the control without IPR (**), and smaller than the control with IPR (***), at $p < 0.005$, $n = 6$. In connective tissue cells, no statistical differences are detected between values.

gland.

Since the organ culture system is separated from the influences of systemic neuronal and hormonal stimulations, it is unlikely that the spontaneous acinar proliferation during the 48 hr-culture period is dependent on exogenous β -adrenergic stimulation. Possibly, it may be dependent on some paracrine-autocrine factors produced in the explants, or on undefined growth factors derived from fetal calf serum. The mechanisms by which these factors cause the proliferation of acinar cells may involve

the activation of CREB. Although CREB was originally discovered as a transcription factor activated by the cAMP/PKA pathway [15, 39], recent studies have indicated that CREB is also activated by protein kinase C [39], Ca/calmodulin-dependent kinase [11, 32] and Ras/MAP kinase [13, 20] pathways, as well as by a steroid hormone estrogen [40]. Thus, the present inhibition of the spontaneous acinar proliferation by antisense-CREB ODN can be interpreted as indicating that CREB functions as the common transcription factor in the multiple signal trans-

duction pathways leading to the proliferation of acinar cells. However, another interpretation, i.e., inhibition of CREB expression in connective tissue cells impairs the production of paracrine growth factors or other kinds of mesenchyme-epithelial interaction that are necessary for acinar cell proliferation, cannot be ruled out completely.

The mechanisms by which activation of CREB causes proliferation of acinar epithelial cells remain to be investigated. Administration of IPR to mice and rats is known to cause a rapid induction of the expression of a protooncogene *c-fos* in the parotid and submandibular glands [5, 24]. *C-fos* expression is also induced in growth factor-stimulated 3T3 cells [17] and in hepatocytes after partial hepatectomy [25], implying the critical role of *c-fos* in cell proliferation. *C-fos* contains CRE in its promoter region [29] and can be induced for transcription by activated CREB [11, 14]. Fos, the product of *c-fos*, forms complexes with another protooncogene product Jun and functions as a transcription factor in itself [3]. These lines of evidence raise the possibility that Fos plays a crucial role in linking CREB activation and acinar cell proliferation. Another candidate protein that may be induced by CREB and function in acinar cell proliferation is the proliferating cell nuclear antigen (PCNA). PCNA is a cofactor of DNA polymerase δ [8] and has been shown to be essential for cell cycle progression in mammalian cells by antisense-inhibition experiments [22, 27]. PCNA gene has a CRE-like sequence in its promoter region and can be induced for transcription by activated CREB [26]. In the system of liver regeneration following partial hepatectomy, some investigators speculate that PCNA induced by activated CREB is involved as causative factor in hepatocyte proliferation [19, 33]. This might be the case also in acinar proliferation in rodent salivary glands.

The present study has demonstrated the effectiveness of the organ culture of neonatal rat submandibular gland in analyzing acinar cell proliferation. In addition to the antisense-inhibition experiments, the pharmacological experiments modulating various signal transduction pathways in this system will give more insights into the mechanisms underlying the postnatal growth and differentiation of submandibular gland.

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VI. References

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