Induction of Sca-1 via activation of STAT3 system in the duct cells of the mouse submandibular gland by ligation of the main excretory duct

Nunuk Purwanti,1 Mileva Ratko Karabasil,1 Shinsuke Matsuo,1 Gang Chen,1,2 Purevyaj Javkhlan,1 Ahmad Azlina,1 Takahiro Hasegawa,1 Chenjuan Yao,1 Tetsuya Akamatsu,1 and Kazuo Hosoi1

1Department of Molecular Oral Physiology, Institute of Health Biosciences, The University of Tokushima Graduate School, Kuramoto-cho, Tokushima-shi, Tokushima, Japan; and 2Department of Occupational Medicine and Environmental Toxicology, School of Public Health, Nantong University, Nantong, China

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To examine the very initial step that takes place immediately after tissue injury and is linked to tissue regeneration, we employed the submandibular gland (SMG), which was injured by ligation of its main excretory duct (MED). Ligation of the MED of the SMG in mice induced the expression of Sca-1, a protein marker of hematopoietic stem cells. In the normal gland, a low level of Sca-1 was expressed, which was localized predominantly in the excretory duct cells. At 1 day after ligation, Sca-1 expression increased prominently in almost all of cells in the duct system, but not in the acinar cells. The level of Sca-1 mRNA had begun to increase at 6 h after ligation and continuously rose thereafter until it reached a plateau, which occurred ~12 h after ligation. STAT3 phosphorylated at its tyrosine-705 (p-STAT3) bound to the GAS element in the ligated gland increased immediately after ligation, and it was localized in the nuclei of all duct cells. The results of an EMSA revealed the specific binding of a nuclear extract to the sequence of the γ-interferon activation site (GAS) present in the Sca-1 promoter and confirmed that such binding increased after ligation. Thus the present study suggests that STAT3, having been phosphorylated following MED ligation, was transferred to the nucleus, where it bound to the GAS element in the promoter of Sca-1 gene, resulting in promotion of Sca-1 gene expression. Actual prevention of STAT3 phosphorylation reduced the ligation-induced Sca-1 elevation.

tissue injury leads cells to proliferate and differentiate to replace dead cells, and inflammatory reactions play a central role in this process (29). β-Cell regeneration is induced by pancreatectomy, but such regeneration does not occur in the absence of inflammation (15). In the case of pancreatic duct obstruction, leukemia inhibitory factor, LIF, a member of the interferon family, plays an important role in the control of proliferation of pancreatic duct cells (3).

It was reported previously that experimental ligation of the main excretory duct (MED) of the salivary gland causes the apoptosis of acinar (Ac) cells and the proliferation of duct cells in both rats and mice (8, 27, 30). Moreover, reopening of the ligated MED induces the repopulation and morphological recovery to the normal state in the submandibular gland (SMG) of rats (1, 28).

Intercalated duct (ID) cells in the developing gland are considered to serve as the stem cells for the striated duct (SD), granular convoluted tubule (GCT), and Ac cells, but this idea has not yet been completely established. Some of the SD cells are also considered to be progenitor of GCT cells, since these cells have the highest susceptibility of thymidine labeling in the mouse submandibular gland (5).

Stem cell antigen-1 (Sca-1) is an 18-kDa glycosylphosphatidylinositol-anchored cell-surface protein or GPI-AP of the Ly6 gene family in mice and is the most common marker used to enrich adult murine hematopoietic stem cells. Besides being found in the hematopoietic system, Sca-1 is also expressed in the cells typed as stem, progenitor, or differentiated cells in a wide variety of tissues and organs (9).

Therefore, Sca-1 would be expected to be one of the key proteins to play an important role during proliferation and differentiation of tissues. When tissues are injured, they are healed by regenerating themselves. Since the cascade involved in the proliferation of duct cells after obstruction of the salivary gland MED is not fully understood yet, in the present study we focused on the initial process triggered by the inflammatory reaction and found it to be linked to the induction of Sca-1, one of the proliferation-related proteins.

MATERIALS AND METHODS

Reagents. Aprotinin, collagenase, pepstatin A, bovine serum albumin (BSA), lipopolysaccharide (LPS), propidium iodide (PI), 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI), and ribonuclease A (RNase A) were bought from Sigma (St. Louis, MO). Superscript one-step RT-PCR was obtained from Invitrogen (Carlsbad, CA). Leupeptin was obtained from Peptide Institute (Osaka, Japan); and a Bio-Rad protein assay kit was from Bio-Rad Laboratories (Hercules, CA). Medium electroendosmosis (ME) agarose and phenylmethylsulfonyl fluoride (PMSF) were products of Wako Pure Chemicals (Osaka, Japan). Complete EDTA-free protease inhibitor cocktail tablets and DIG luminescent detection kit for nucleic acids were purchased from Roche Diagnostics (Mannheim, Germany). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG, enhanced chemical luminescence (ECL) detection kit, and Amersham Hybond-N+ were obtained from GE Healthcare (Buckinghamshire, UK). Fluorescein isothiocyanate (FITC)-conjugated affinity-purified rabbit anti-rat IgG (H+L) and FITC-conjugated affinity-purified goat anti-rabbit IgG were purchased from Vector Laboratories (Burlingame, CA). HRP-conjugated affinity-purified goat anti-rat IgG came from Chemicon International (Temecula, CA). Texas red-conjugated affinity-purified goat anti-rabbit IgG (H+L) and Texas red-conjugated affinity-purified sheep anti-mouse IgG (H+L) were products of Jackson ImmunoResearch Laboratories (West Grove, PA). Purified rat...
anti-mouse Sca-1 monoclonal antibody was bought from BD Biosciences Pharmingen (San Diego, CA). NuSieve and SeaKem agarose were purchased from Cambler Bio Science (Rockland, ME). Nembrubit (pentobarbital sodium) was obtained from Abbott Laboratories (North Chicago, IL) and Fuji RX X-ray film from Fuji Film ( Kanagawa, Japan). Tissue-Tek optimum cutting temperature compound was from Sakura Finetechanical (Tokyo, Japan). 3-Aminopropyltriethoxysilane (APS)-coated microslide glasses and microcoverglasses were products of Matsunami Glass (Osaka, Japan). Rabbit polyclonal antibody against signal transducer and activator of transcription 3 (STAT3) (C-20) and STAT3 (C-20)X TransCruz were bought from Santa Cruz (Santa Cruz, CA). Rabbit polyclonal antibody against phospho-STAT3 tyrosine-705 (p-STAT3) and U0126 were products of Cell Signaling Technology obtained via New England Biolabs (Hitchin, Hertfordshire, UK). AG490 and JSI-124 (Cucurbitacin I) were obtained from Calbiochem (La Jolla, CA).

Animal experiments. C57BL/6 male mice of 8 wk of age were used throughout the present experiments. They were obtained from Japan SLC (Shizuoka, Japan) and maintained in our animal facility under standard conditions (12:12-h light-dark cycle) with free access to food and water. Mice were anesthetized with Nembutal (sodium pentobarbital, 50 mg/kg body wt ip), and the MED of the left SMG was homogenized in eight volumes of the same buffer by using a Dounce homogenizer (Wheaton Science Products, Millville, NJ). The SMG was homogenized in nine volumes (wt/vol) of 50 mM Tris·HCl (pH 7.5), 0.2 mM sodium pyrophosphate, 1 mM PMSF, 10 mM MgCl₂, 20 mM potassium phosphate buffer (pH 6.8), 1 mM Na₃VO₄, and 50 μM Na₂MoO₄ (buffer A). The tissue was homogenized in eight volumes of the same buffer by using a Dounce homogenizer (Wheaton Science Products, Millville, NJ). The homogenate was filtered through 2 layers of cheesecloth and centrifuged at 1,000 g for 20 min. The resulting white clear pellet (the nuclear pellet) was suspended in a minimum volume of ice-cold buffer A, and homogenized again with the Dounce homogenizer. Next, the resulting suspension was mixed with 3.5 volumes of ice-cold buffer consisting of 2.4 M sucrose, 10 mM MgCl₂, 10 mM potassium phosphate buffer (pH 6.8), 1 mM Na₃VO₄, and 50 μM Na₂MoO₄ (buffer B) and centrifuged at 100,000 g for 1 h. The white clear pellet (the nuclear fraction) was suspended in buffer consisting of 0.25 M sucrose, 20 mM Tris·HCl (pH 7.5), 5 mM MgCl₂, 1 mM Na₃VO₄, and 50 μM Na₂MoO₄ (buffer C) at a ratio of 500 μl of buffer per gram original wet weight of the liver. The protein concentrations of samples thus obtained were measured by performing a Bio-Rad protein assay using BSA as the standard.

Western blotting. The protein sample prepared as described above was subjected to Western blot analysis following the standard procedure using a Mini-Protein Electrophoresis apparatus (Bio-Rad Laboratories). The protein amounts applied for analysis were the following: 1 μg for analysis of kallikrein mK22 and Na⁺-K⁺-ATPase α-subunit, 5 μg for analysis of AQP5, 5 or 10 μg Sca-1, and 20 μg (both the SMG homogenate and liver nuclear fraction) for analysis of STAT3 and p-STAT3. AQP5, kallikrein mK22, STAT3, and p-STAT3 were resolved by performing 12% reducing SDS-PAGE, whereas 7.5% reducing SDS-PAGE was used for Na⁺-K⁺-ATPase α-subunit. Sca-1 was analyzed by 15% nonreducing SDS-PAGE.

After electrophoresis, the proteins were transferred onto a nitrocellulose membrane by means of a Mini Trans Blot apparatus following the standard procedure. The membrane was blocked with either 3% (for AQP5, Na⁺-K⁺-ATPase α-subunit, and Sca-1) or 5% skim milk (for kallikrein mK22, STAT3, and p-STAT3) at room temperature for 2 h. All membranes were incubated with each primary antibody diluted in their respective blocking solution at 4°C. The dilutions of primary antibodies used were as follows: anti-AQP5, 1:3,000; anti-kallikrein mK22, 1:10,000; anti-Na⁺-K⁺-ATPase α-subunit, 1:300,000; anti-Sca-1, anti-p-STAT3, and anti-STAT3, 1:1,000. For control reactions, membranes were incubated with the same concentrations of antisera preabsorbed with the respective antigen used for immunization or with the relevant normal serum. Membranes were washed and subsequently incubated at room temperature for 1 h with 3,000 times-diluted HRP-conjugated anti-rabbit IgG (for AQP5, kallikrein mK22, Na⁺-K⁺-ATPase α-subunit, STAT3, and p-STAT3) or with 5,000 times-diluted HRP-conjugated anti-rat IgG (for Sca-1 analysis). All membranes were subjected to ECL reaction and exposed to an X-ray film for the appropriate time. The expression level of each protein was quantified by densitometry analysis using the NIH Image software.

For analysis of small-size STAT3, the homogenate of the SMG from a MED-ligated animal and the liver nuclear pellet prepared as described above were mixed and incubated at 37°C for 30 min. The mixture was then analyzed by Western blotting using anti-STAT3 and anti-p-STAT3 antibodies.

Immunohistochemistry. Mice were anesthetized and perfused with the fixative to fix the SMG tissue, which was dissected and processed for immunohistochemistry as described previously (20). Frozen sections were cut at a 5-μm thickness and mounted on APS-coated slide glasses. These tissue sections were postfixed in 100% ethanol at −20°C for 1 min and washed with PBS. For single immunostaining, sections were blocked with either 5% goat serum

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(for kallikrein mK22 and Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit) or 1.5% goat serum (for AQPS) or 5% rabbit serum (Sca-1 staining) in PBS. Tissue section was homogenized in buffer consisting of 50 mM Tris·HCl (pH 8.0), 280 mM NaCl, 0.05% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.5 mg/ml leupeptin, 3 mg/ml aprotinin, 1 mg/ml pepstatin, and 0.1 mM Na\(_3\)VO₄. The tissue sections were prepared from the MED-ligated mice. Mice were euthanized at 0, 1, 3, 6, 12, and 24 h after ligation, total RNA of the SMG was isolated by the use of Tri-Reagent as described previously (33). The RT-PCR was carried out at 45°C for 30 min, followed by amplification of cDNAs of IL-6, IL-1, β-actin and Sca-1 amplification. All thermal cycle conditions, including primer sequences for amplification of the cDNAs of IL-6, IL-1β, TNF-α, 35 cycles of PCR amplification were employed. All thermal cycle conditions, including annealing, denaturation, and extension, were the same as those for β-actin and Sca-1 amplification.

All RT-PCR reactions were performed in a TaKaRa PCR Thermal cycler MP, model TP3000 (Shiga, Japan), and RT-PCR products were resolved by electrophoresis in 3% agarose gel (NuSieve:SeaKem = 3:1) and 1.5% ME agarose gel. The primer sequences for amplification of the cDNA are listed in Table 1.

Table 1. Primer sequences for amplification of the cDNAs of inflammatory cytokines, Sca-1, and β-actin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Product Size, bp</th>
</tr>
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<tbody>
<tr>
<td>IL-6 Sense</td>
<td>5′-GCAAAACTCTGATATAATCAGAAAGATC-3′</td>
<td>337</td>
</tr>
<tr>
<td>IL-1β Sense</td>
<td>5′-GTGATTTTTGCCAGATGATCCTC-3′</td>
<td>418</td>
</tr>
<tr>
<td>TNF-α Sense</td>
<td>5′-ATGCAGACAGAAAGATGATC-3′</td>
<td>305</td>
</tr>
<tr>
<td>Sca-1 Sense</td>
<td>5′-GCCACTTTGTGGTTTGCTGAG-3′</td>
<td>403</td>
</tr>
<tr>
<td>β-Actin Sense</td>
<td>5′-ACCCACACTGTGCCCATCTA-3′</td>
<td>289</td>
</tr>
</tbody>
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EMA. EMSAs were performed by following the procedure of Khan et al. (13) with slight modification. The crude protein extracts of the SMG were prepared from the MED-ligated mice. Mice were euthanized at 0 and 6 h after MED ligation, and the SMG dissected was homogenized in buffer consisting of 50 mM Tris-HCl (pH 8.0), 280 mM NaCl, 0.05% Nonidet P-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, 0.5 mg/ml leupeptin, 3 mg/ml aprotinin, 1 mg/ml pepstatin, and 0.1 mM Na\(_3\)VO₄. The homogenate was passed through two layers of cheesecloth, and the filtrate obtained was directly used for EMSA. The protein concentration of the sample was measured by performing a Bio-Rad protein assay, using BSA as the standard, and the sample was subjected to EMSA as described below.

The double-stranded oligonucleotide corresponding to the γ-feron activation site (GAS) element has the sequence shown below (13), which was labeled with DIG (5′-CATGTATGCTATTTCTGTAAAGTG-3′ 3′-AACCTGATTAAGGCATCTGACCTGAC-5′ custom ordered from Hokkaido System Science, Hokkaido, Japan). Eight micrograms of crude extract were mixed with 1 ng of DIG-labeled oligonucleotide in 11.5 µl gel mobility shift buffer that comprised 20 mM HEPES (pH 7.9), 4% Ficoll, 1 mM MgCl₂, 0.4 mM KCl, 0.1 mM EGTA, and 0.5 mM DTT. After 10 min of preincubation on ice, the binding reaction was carried out at room temperature for 20 min. For the competition and supershift assay to ensure the specific binding of STAT3 to the GAS element, a 50 molar excess of unlabeled oligonucleotide and 1 µg of anti-STAT3 (C-20)X Trans Cruz antibody (qualified for supershift assays) was added 10 min before the addition of the DIG-labeled oligonucleotide. Ten microliters of the reaction mixture were resolved in 4% non-denaturing polyacrylamide gel in Tris-borate EDTA buffer (TBE). After electrophoresis, protein-DNA complexes were transferred onto a nylon membrane (Amersham Hybond-N⁺) by using the semidy procedure (12) with 2 mA/cm² for 1 h. Transferred protein-DNA complexes were fixed by UV cross-linking at 120,000 µJ/cm² for 5 min. After having been washed with maleic acid buffer containing 0.3% Tween 20, the membrane was incubated with the blocking solution for 30 min, followed by continued incubation with anti-digoxigenin-AP for 30 min. The membrane was equilibrated for 5 min with CSPD solution (DIG luminescent detection kit for nucleic acids) and exposed to an X-ray film for an appropriate time to visualize the chemiluminescence of the shifted band.

RESULTS

Effects of ligation of the MED of the SMG on the expression of AQPS, kallikrein mK22, Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit, and Sca-1. To analyze the effects of ligation of the MED of the mouse SMG on the cellular marker in each component of the SMG, we performed Western blotting to analyze the expression levels of AQPS, kallikrein mK22, and Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit. The former two proteins are markers of Ac and GCT cells, respectively, whereas the last one is expressed in GCT, SD, and ID cells. The AQPS and kallikrein mK22 of the SMG were detected as 27-kDa protein bands, whereas the Na\(^{+}\)-K\(^{+}\)-ATPase α-subunit was detected as a 100-kDa protein band. Sca-1, a stem cell marker, was
detected as an 18-kDa band (Fig. 1B). All of these bands almost completely disappeared when the primary antibody was substituted with the peptide-preabsorbed antibody or normal serum, indicating the specificity of the immunoreactions (Fig. 1B).

As shown in Fig. 1, A and C, the expression of AQP5, kallikrein mK22, and Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit decreased gradually following ligation of the MED of the SMG. The reduction in the levels of kallikrein mK22 and Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit was significant at day 3 after ligation, whereas that for AQP5 was significant at day 6 (Fig. 1, A and C). In the contralateral gland, the expression of AQP5, kallikrein mK22, and Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit increased slightly (Fig. 1, A and C); although small, the increase was reliable as such elevation was not observed in the SMG from sham-operated mice. The changes seen in the contralateral gland may have been caused by a (putative) humoral factor(s) or neural control from the injured gland or by any other mechanism. The precise mechanism is, however, obscure at present. The expression of Sca-1 strongly increased at 1 day after ligation in both the ligated and contralateral glands and decreased thereafter. At day 3 after ligation, the expression of Sca-1 in either gland was still higher than that at day 0 (Fig. 1, A and C).

**Immunohistochemical changes in expression of AQP5, kallikrein mK22, Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit, and Sca-1 in the SMG following MED ligation.** Immunohistochemical staining showed that ligation of the MED of the SMG induced changes in cell morphology as well as in immunohistochemical expression of AQP5, kallikrein mK22, and Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit in duct and Ac cells (Fig. 2A). On the first day after ligation, SMG morphology did not show any difference from that on day 0 (control) and apparently appeared to be normal. The expression of AQP5 was strong in the apical and lateral membranes of Ac cells, and kallikrein mK22 was expressed in the duct cells, whereas the Na\(^+\)-K\(^+\)-ATPase \(\alpha\)-subunit was strongly expressed at the basolateral membrane of duct cells and weakly expressed by Ac cells (Fig. 2A). At 3 days after
ligation, the number of Ac cells expressing AQP5 decreased appreciably, and a similar change was observed in the cells in which kallikrein mK22 and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase α-subunit were expressed. By 6 days after ligation, the Ac cells had become obscure, although AQP5 was still expressed in a limited part of them (green stain in photo of experimental gland, day 6, AQP5 stain in Fig. 2A). The diameter of the duct cells appeared to be reduced, and some of the duct cells had a dilated lumen. Low levels of kallikrein mK22 and Na\textsuperscript{+}-K\textsuperscript{+}-ATPase α-subunit were still expressed in the duct cells at day 6 (Fig. 2A).

The MED ligation induced Sca-1 expression in cells of the duct system. On day 0 (control) Sca-1 expression was detected predominantly in excretory duct cells. The Sca-1 expression increased prominently in almost all duct cells on the first day after ligation (Fig. 2A). At 3 days after ligation, the number of cells that expressed Sca-1 did not change, but the level of Sca-1 expression in each cell decreased. The number of Sca-1-positive cells was lower at 6 days after ligation. These results are in good agreement with the Western blotting data.

We next verified the exact location of Sca-1 induced by MED ligation by conducting a double immunostaining experiment (Fig. 2B). On the first day after ligation, Sca-1-positive immunoreactions appeared in the almost all cells in the duct system, which cells were definitely distinguished from Ac cells, as shown in the merge view of Sca-1 and AQP5. The cytoplasmic area of GCT cells, which showed positive reaction of kallikrein mK22, was also positive for Sca-1, resulting in the appearance of yellow signals in the merged image (Fig. 2B). Similarly, basal cell membrane of GCT/SD cells were positive for both Na\textsuperscript{+}-K\textsuperscript{+}-ATPase α-subunit and Sca-1, as obviously shown by the yellow signals (Fig. 2B). These results indicate that Sca-1 induced by MED ligation was localized in both basal cell membrane and cytoplasm of GCT/SD cells.

**Effects of MED ligation on the mRNA levels of inflammatory cytokines and Sca-1 in the SMG.** We found by RT-PCR that MED ligation increased the expression of mRNAs of inflammatory cytokines IL-6, TNF-α, and IL-1β in the ligated SMG (Fig. 3). Their mRNA levels increased as early as 1 h after ligation. Expression of IL-6 mRNA reached its peak level at 6 h after ligation, whereas TNF-α and IL-1β mRNA levels were continuously high until 24 h after ligation. On the other hand, the Sca-1 mRNA level did not start to increase until 3 h postligation and then continued to rise thereafter. In the contralateral gland, the expression pattern of these three cytokines and Sca-1 mRNAs was similar to that found in the experimental glands; however, the mRNA levels in the contralateral gland were lower than those in the ligated gland (Fig. 3).

To investigate whether the inflammatory cytokines were induced by direct action of ligation or by anesthesia injection or skin injury, we conducted a sham operation as well in this experiment. The mRNAs for these inflammatory cytokines and Sca-1 in the SMG of sham-operated mice were much lower than those in the contralateral and ligated gland (data not shown). These findings suggest that the increased expression of inflammatory cytokines and Sca-1 mRNAs in the ligated SMG was mostly due to the injury induced by the ligation.

**Effects of the MED ligation on the expression of STAT3 and p-STAT3 in the SMG.** It was earlier reported that GAS, a binding element of p-STAT3, is present in the promoter of Sca-1 (13) and that JAK-STAT signaling system is triggered by IL-6. We therefore examined the STAT3 expression and its phosphorylation following MED ligation. By Western blotting, we found that the molecular weights of SMG STAT3 and p-STAT3 were 40 kDa (Fig. 4A). This molecular mass was smaller than that reported for the liver STAT3/p-STAT3 (97.4 kDa, Ref. 14). The band of p-STAT3 completely disappeared when the antibody was preabsorbed with p-STAT3 blocking peptide, indicating that the positive band was due to a specific reaction and that the molecular mass of SMG STAT3 was lower than the reported size.

To investigate how this small-size STAT3 was produced, we examined the possible involvement of salivary gland proteases in its formation. We mixed a nuclear pellet prepared from the liver (expressing 97.4 kDa STAT3) from an LPS-injected mouse and the homogenate of a SMG from a MED-ligated mouse. In the presence of the total SMG homogenate, the liver STAT3 was degraded, and the intensity of the 40-kDa band increased compared with that for the sample without the liver nuclear pellet sample (Fig. 4B). Also in the ligated SMG, anti-pSTAT3 antibody, although weakly, clearly detected the 92-kDa band by Western blotting, and this band completely disappeared when the anti-pSTAT3 antibody was preabsorbed with peptide antigen (Fig. 4A). All these results suggest that the small-size STAT3 in the SMG was proteolytically produced from 97.4-kDa STAT3 by SMG proteases.

**MED ligation induced phosphorylation of STAT3 and localization of p-STAT3 in the SMG.** Next, we examined the time course of phosphorylation of STAT3 in the ligated and contralateral glands. The level of p-STAT3 in both the ligated and contralateral SMGs increased immediately at 1 h after ligation, becoming maximal in the ligated gland at 6 h after ligation and decreased thereafter (Fig. 5, A and B). The peak level of p-STAT3 in the contralateral gland appeared a little shifted (12 h). Ligation did not alter the expression level of STAT3 (Fig. 5A).

Immunohistochemical staining showed that the level of p-STAT3 expression was very low in duct cells in the control SMG (0 h). However, at 6 h after ligation many nuclei in the duct cells of the MED-ligated gland became strongly positive for p-STAT3 (Fig. 5C). Positive reactions were scarcely observed in the Ac cells. In the contralateral gland, some of the nuclei, but not all, in the duct cells became positive for p-STAT3; thus the number of nuclei in the p-STAT3-expressing duct cells of the contralateral gland was less than that in the ligated gland. Expression of p-STAT3 was scarcely observed in the nuclei of Ac cells in the contralateral gland as well. No positive reaction appeared when p-STAT3 antibody had been preabsorbed with p-STAT3 blocking peptide, indicating that the positive staining was due to a specific reaction (Fig. 5C).

**Effects of JAK-STAT and MEK1/2 inhibitors on the STAT3 and p-STAT3 expression and elevation of Sca-1 in the MED-ligated SMG.** To explore the signaling pathway inducing the phosphorylation of STAT3 at its tyrosine-705, we examined the effects of various inhibitors, first using AG490 and JSI124, which are inhibitors of the JAK-STAT pathway. AG490 and JSI124 are specific and potent JAK-2 protein tyrosine kinase inhibitors, which inhibits the JAK/STAT3 pathway and suppresses cell growth in vitro and tumor growth in vivo (2, 19). As shown in Fig. 6A, the administration of AG490 inhibited the phosphorylation of STAT3 at tyrosine-705; whereas JSI124, another JAK-STAT inhibitor, did not (Fig. 6A).
Since there are several reports describing that STAT3 is also activated by MEK-ERK signaling to generate p-STAT3 (7, 14, 22, 25), we next investigated the effects of U0126. U0126 is known to inhibit MEK-1 and MEK-2, strongly preventing the ERK1/2 pathway (6). We injected U0126 at 1 h before ligation. Similar to the results of the JSI124 inhibitor experiment, there was no inhibitory effect by U0126 on the production of p-STAT3 (Fig. 6B). Sun et al. (26) showed that combined treatment of skeletal muscle stem cell-derived myoblasts with JAK-siRNA and U0126 in vitro gave more potent inhibition of JAK1 than either single treatment provided. We therefore hypothesized that both JAK-STAT and MEK/ERK1/2 pathways may be involved in additive generation of p-STAT3. Thus we injected both JSI124 and U0126 at 1 h before ligation. We found that concomitant injection of JSI124 and U0126 inhibited the formation of p-STAT3 (Fig. 6B). Such inhibition by these two inhibitors was more clearly demonstrated in the contralateral gland (Fig. 6B). Although this is still preliminary and needs to be confirmed, the present study indicates that both JAK-STAT and MEK/ERK1/2 pathways may be involved in the generation of p-STAT3 in the MED-ligated SMG.

We next examined the effects of JAK-STAT and MEK1/2 inhibitors on the ligation-induced elevation of Sca-1. Sca-1 protein levels in the SMG were elevated at 10 h after ligation, although the level was lower than those seen in the same tissue at 24 h after ligation. As shown in the Fig. 7, single injection
of AG490 and combined administration of JSI124 and U0126 significantly reduced the ligation-induced Sca-1 elevation. This experiment, along with other data shown above, confirmed that the STAT3 pathway is involved in the ligation-induced Sca-1 elevation and that p-STAT3 generation was indispensable for Sca-1 induction.

Binding of STAT3 to the GAS element in the Sca-1 promoter.

To determine whether p-STAT3 could bind to the GAS element in the promoter of Sca-1 gene, we prepared crude extracts of the SMG from the normal and MED-ligated gland (6 h after ligation) and analyzed them by EMSA. The results showed the appearance of a shifted band of the protein-GAS complex in both normal and ligated glands, and the intensity of the shifted band in the ligated gland was stronger than that in the normal gland (Fig. 8). The band shift was not observed for the crude sample without probe or for the binding mixture without the crude protein extract. The protein-GAS complex obviously disappeared in a competition assay, when the crude protein extract was preincubated with a 50-fold molar excess of unlabeled GAS probe before the addition of the labeled-GAS probe. Furthermore, in the supershift assay, anti-STAT3 antibody reduced formation of the protein-GAS complex. The result of these competition and supershift assays demonstrate the presence of p-STAT3 binding to the GAS element of Sca-1 promoter in the SMG, which binding was increased by duct ligation.

DISCUSSION

In the present study, we observed that the expression levels of AQP5, kallikrein mK22, and Na⁺-K⁺-ATPase α-subunit decreased at 3 and 6 days after MED ligation. Since it has already been established that ligation of the SMG induces Ac apoptosis (8, 27, 30), a decrease in the AQP5 level would be due to such an alteration. On the other hand, decrease in the levels of kallikrein mK22 and Na⁺-K⁺-ATPase α-subunit may have been caused by progressive excretory dysfunction of duct cells induced by ligation, as reported by Martinez et al. (16).

The level of Sca-1 expression, on the other hand, increased strongly in many duct cells at 1 day after ligation. This finding indicates that the experimental injury stimulated Sca-1 expression. Wang et al. (31) reported that Sca-1⁺/CD31⁻ cells increases in number at 7 days after a myocardial infarction. Such an increase may play a pivotal role in normal cardiac repair and remodeling. Previous studies indicated that ligation of the MED of the SMG induces the proliferation of duct cells (8, 27, 30). Our results showed that the ligation triggered Sca-1 expression in all cell types of the duct system, implying that Sca-1 may have an important role in the proliferation of most cells in this system.

Since stem cells of the SMG have not been established yet, expression of Sca-1 in many cells in the duct system of the salivary gland would imply that the differentiated cells may have dedifferentiated and proliferated to induce a rapid recovery of the gland when severe damage occurs (5, 11). Similar evidence was reported in the case of liver regeneration after injury; i.e., the mature cells play important roles during regeneration. When the proliferation ability of mature cells is diminished, the liver progenitor cells are activated (21). Denny et al. (5) reported that ~70% of GCT cells are renewed by self-proliferation and the rest by differentiation from ID and SD cells. In the present experiment, we showed that Sca-1 was not expressed in the Ac cells. It is conceivable that Ac cells would not enter into the proliferation phase in response to ligation, probably because Sca-1 did not become expressed. This idea is consistent with the known fact that ligation induces apoptosis of Ac cells.

The distribution of cells in tissues is determined by the equilibrium among proliferation, differentiation, and programmed cell death. This balance will be altered during tissue injury and regeneration. An inflammatory reaction is triggered...
by tissue injury, and cytokines generated would be involved in the regeneration process (24). In our study, we found that ligation of the MED of the SMG increased the expression of inflammatory cytokines, including IL-6, in the SMG. MED ligation of the SMG led to a transient increase in the IL-6 mRNA level, which reached a peak level at 6 h, whereas the expression of Sca-1 mRNA just started to increase at 6 h after ligation. IL-6, as one of the proinflammatory cytokines, has been reported to induce the expression of Ly-6A/E (Sca-1) in T cells (4). Thus the sequential elevation of Sca-1 in the duct-ligated SMG shown in this study was considered to be induced after the transient increase in the IL-6 level.

The Sca-1 mRNA and protein levels increased in both the experimental and contralateral glands. Elevation of the IL-6 mRNA level in the contralateral gland as well as in the experimental gland upon MED ligation suggests that the increase in the level of this inflammatory cytokine triggered the initial step of Sca-1 induction. This fact also implies that the inflammation cytokine(s) released in the experimental gland may have also affected the contralateral gland via circulation, although the details are still unknown.

IL-6 in the developing mouse submandibular gland plays an important role in the induction of cell proliferation and suppression of apoptosis (18). IL-6 will bind to the cognate receptor, IL-6R, and gp130 to activate the JAK-STAT pathway in parallel with activation of the RAS/MAPK pathway to induce cell proliferation (18). STAT3 plays critical roles in gp130-mediated cell proliferation by inhibiting apoptosis and in the transition from the G1 and S phase in the cell cycle (7). Activation of STAT3 requires phosphorylation of its tyrosine-705 to facilitate translocation of its signal to the nucleus, where it binds to the promoter region of the target genes (32).
results show that MED ligation of the SMG induced phosphorylation of STAT3 and that the p-STAT3 level reached its peak at 6 h after ligation, which time was similar to the time course of IL-6 mRNA elevation; the Sca-1 mRNA then started to rise. p-STAT3 became localized in the nuclei of duct cells, but not in those of the Ac cells. These results imply possible roles of p-STAT3 in the Sca-1 induction in the duct cells. Actually, treatment of experimental animals with inhibitors for JAK-STAT and ERK1/2-STAT pathways (AG490 and JSI124 plus U0126, respectively) prevented the Sca-1 elevation by ligation.

In our study, we found that the molecular weight of STAT3 and p-STAT3 in the SMG was smaller than that reported for liver STAT3 and suspected that this small-size STAT3 protein in the SMG was converted by proteases in the SMG (a larger size cDNA than would be expected for a 40-kDa protein) was synthesized and processed in the SMG. 

STAT3 protein in the liver was degraded when the liver sample was incubated with the homogenate of SMG obtained from MED-ligated mice. Thus it is very probable that the small-size STAT3 in the SMG was converted by proteases in the SMG from full-size STAT3α, although further investigations are required to examine this phenomenon. In preliminary experiments using the total RNA from the SMG, a 1,740-bp cDNA (a larger size cDNA than would be expected for a 40-kDa protein) was detected by RT-PCR, implying the possibility that the large-size STAT3 was synthesized and processed in the SMG.

The GAS element, a small palindrome consensus sequence that can be bound by the p-STAT3 protein, is present in the promoter region of the Sca-1 gene. Binding of p-STAT3 to this GAS element was reported to induce Ly-6 A/E (Sca-1) expression in T cells (13, 4). In our study, we found that MED ligation of the SMG increased the GAS-binding activity. The supershift experiment implied that STAT3 (probably p-STAT3) was responsible for this binding. Binding of STAT3 (p-STAT3) to GAS element in the promoter of Sca-1 gene would induce Sca-1 expression. 

In conclusion, the following mechanism is proposed from the present study: i.e., ligation of the MED of the SMG induces IL-6 expression. IL-6 binds to its receptor to trigger a kinase to phosphorylate STAT3 at its tyrosine-705. p-STAT3 is next transferred to the nucleus, where it supposed binds to the GAS element in the promoter region of the Sca-1 gene to induce Sca-1 expression. Induced Sca-1 may be one of key molecules involved in the regulation of duct cell proliferation.

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**Fig. 6.** Effects of inhibitors of JAK-STAT and ERK1/2-STAT pathways on phosphorylation of STAT3. A: effects of JSI124 and AG490. JSI124 (1 mg/kg body wt ip) or AG490 (40 mg/kg body wt ip) was injected into mice 1 h before ligation. The mice were then killed at 6 h after ligation. The SMG was dissected and subjected to Western blotting using 20 μg of the SMG homogenate to detect STAT3 and p-STAT3. B: effects of U0126 and JSI124. The experimental conditions were the same as those described in A, except 30 mg/kg body wt of U0126 was given singly or in combination with 1 mg/kg body wt of JSI124. All reagents were dissolved in DMSO. Blots shown are representative of 2 other series of samples. Vehicle, DMSO.

**Fig. 7.** Effects of inhibitors of JAK-STAT and ERK1/2-STAT pathways on ligation-induced Sca-1 elevation. AG490 (40 mg/kg) and JSI124 plus U0126 (1 mg/kg and 30 mg/kg ip, respectively) were injected 30 min before ligation. Mice were killed at 10 h (Lig1) or 24 h (Lig2) after ligation, and samples for Sca-1 assay were prepared as described. Ten micrograms of sample proteins were subjected to analysis. Other experimental conditions are described in the text. NO, no operation; Lig1, the gland at 10 h after ligation; Sham, sham operation; Lig2, the gland at 24 h after ligation; None, no injection; Veh, vehicle; AG, AG490; JSI+U, JSI124 plus U0126.

**Fig. 8.** Detection of the binding of nuclear extracts to the γ-interferon activation site (GAS) element. A crude nuclear extract of the SMG was obtained at 6 h after ligation, and EMSA was carried out by using a DIG-labeled GAS probe. Samples applied were the following (from left to right): crude nuclear extract of the SMG without probe; reaction mixture without crude nuclear extract; protein-GAS shift assay for the SMG at 0 h after ligation; protein-GAS shift assay for the SMG at 6 h after ligation; competition assay in the presence of a 50-fold molar excess of unlabeled probe; supershift assay in the presence of 1 μg of anti-STAT3 antibody. The specific binding of the protein-GAS probe is indicated by the arrowhead.

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