Effects of Stress Response to Surgical Procedures upon Secretion of Salivary Immunoglobulin A in Mice

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Abstract: Secretory immunoglobulin A (sIgA) plays an important role in mucosal immunity, and salivary levels change in response to social, psychological and physical stress. However, little is understood about how surgical stress affects salivary sIgA. The results of the present study showed that mouse salivary IgA concentration was significantly elevated immediately after surgery and returned to pre-surgical levels after 24 h. Thus, the surgery did not suppress IgA secretion under our experimental conditions, suggesting that mucosal immunity was not perturbed. Since the role of α-adrenergic receptors involved in IgA secretion has remained unclear under surgical stress, we further examined the effects of either α₁-adrenergic (prazosin) or α₂-adrenergic antagonist (yohimbine) on the salivary sIgA. Yohimbine, but not prazosin, antagonized the surgically induced salivary IgA enhancement, indicating mediation by α₂-receptor stimulation. The mRNA for IgA was not altered in the salivary gland after surgery, suggesting that surgical stress did not stimulate IgA synthesis in the salivary gland cells. In conclusion, it is suggested that the surgical stress does not perturb mucosal immunity in our experimental model, although a transient increase of concentration of salivary IgA was observed immediately after surgical insult.

Key words: Immunoglobulin A, saliva, stress

Introduction
Salivary glands produce and secrete numerous proteins and peptides into the saliva that play roles in host defence and in the maintenance of mucosal tissue¹. Secretory immunoglobulin A (sIgA) acts in mucosal defence, principally by simply binding to soluble or particulate antigens, rendering them non-pathogenic to the mucosal epithelium. Secretory IgA is found in various secretory fluids, including breast milk and saliva, in addition to nasal, gastrointestinal and bronchial secretions.

Salivary sIgA might be a potential marker of stress in humans⁵⁻⁷, dogs⁶ and rats⁷. Prolonged exposure to stress, such as during revision periods prior to university examinations, is associated with reductions in sIgA⁷⁻⁹. On the other hand, sIgA transiently increases in response to acute psychological stress such as public speaking, computer game challenges and mental arithmetic³⁻¹⁰⁻¹¹.

The intensely acute physical stress of surgery stimulates the hypothalamo-pituitary-adrenal (HPA) and
hypothalomo-sympathetic nerve axes. We recently demonstrated that surgical procedures cause alterations in murine salivary secretion. A simple surgical procedure that consisted of a skin incision, formation of an abdominal flap and subsequent suture, increased and decreased salivary protein concentrations and salivary flow, respectively. Changes in the profile of secreted salivary proteins in mice that underwent surgery were significant according to SDS-PAGE.

Cellular immunity is depressed by the neuroendocrine response to surgery that includes reduced neutrophil chemotaxis, decreased lymphocytes and a depressed mononuclear cell function response to PHA or Con-A. On the contrary, humoral immunity is enhanced by surgical procedures. However, little is known about how surgical stress affects mucosal immunity. Although models of stress such as thermal injury, femur fracture, heptectomy and hemorrhagic shock decrease sIgA levels in various secretions including bile, pulmonary secretions and succus entericus, changes in salivary sIgA after surgical stress have not been reported.

To clarify how surgical stress affects mucosal immunity, we compared the levels of salivary IgA between surgically treated and untreated mice. Moreover, we applied a competitive reverse transcription polymerase chain reaction (RT-PCR) to clarify the effects of surgical stress on IgA synthesis in the mouse salivary gland.

The secretion of IgA is controlled by the autonomic nervous system and increased by both sympathetic and parasympathetic nerve stimulation. However, studies of the details of the regulatory mechanisms are still underway. We therefore used α-adrenergic antagonists to clarify the specificity of autonomic receptor stimulation in surgically treated mice with respect to changes in IgA levels.

Materials and methods

1. Animals

Male ICR mice (26 to 40-weeks-old) weighing between 35 and 55 g were housed in groups of 5 - 6 per cage with water and solid laboratory chow (Nippon CLEA, Tokyo, Japan) ad libitum. All experiments were performed in accordance with the National Research Council Guide and were approved by the Committee for Animal Care at Tsurumi University.

2. Surgical procedures

Surgical insult consisted of a skin incision, development of an abdominal flap and suture. All experiments were conducted in the morning.

A straight, 6.5-cm incision was made in the skin under pentobarbital anaesthesia (Abbott, IL, USA: 45 mg/kg i.p.) at the middle of the abdomen and extended to the neck via the pectoral region, and a skin flap was created by blunt dissection of the subcutaneous connective tissue. The wound was closed with 6-0 monofilament nylon sutures. Surgery began 10 min after pentobarbital injection. The surgical procedures were completed within 20 min after pentobarbital injection. Although the flap was extended to the neck region, the salivary glands were not affected by the procedure.

The body temperature of the mice under general anaesthesia became approximately 3°C lower than that in conscious animals. However, saliva collection did not significantly influence body temperature.

Untreated mice were used as a control group for saliva collection under pentobarbital anesthesia.

3. Administration of antagonists

Separate groups of surgically treated animals were injected subcutaneously with α1- or α2-adrenergic antagonists (0.1 mg/kg of prazosin or 10 mg/kg of yohimbine, respectively; Sigma, MO, USA), to study the effect of surgical stress on salivary composition. Prazosin and yohimbine were administered 5 min before pentobarbital anesthesia. Pilocarpine (2 mg/kg)-stimulated saliva was collected as described below.

4. Collection of saliva and salivary glands

Overnight fasted mice underwent the surgical procedure under general anesthesia. Flow rate and composition of the saliva were analyzed.

After surgery, the mice were then intraperitoneally injected with 0.5 mg/kg of pilocarpine (Wako Pure Chemical Ind., Osaka, Japan) to stimulate salivary secretion. Saliva was subsequently collected from the oral cavity over the next 10 min, by micropipet, and it was placed in micro-centrifuge tubes placed in an ice bath. All samples were weighed. Saliva volume was calculated, assuming that 1 ml was equal to 1 g weight of saliva.

After collecting saliva, the parotid and submandibular glands were removed for measurement of IgA mRNA.
levels.

To determine the time course changes, saliva and salivary glands were collected under general anesthesia from the separate groups of animals at different times, on day 1, 7, 14, and 28.

5. Analyses of saliva

The protein content of saliva samples was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard.

The salivary IgA concentration was determined using the Mouse IgA ELISA Quantitation Kit (Bethel Labs., USA). Briefly, 96-well microplates (Sumitomo Bakelite Co., Ltd., Japan) were coated with goat anti-mouse IgA for 60 min. After washing the wells and blocking non-specific binding with 1% BSA, diluted samples and standards were added to the wells and the plates were incubated for 60 min at 37°C. Secondary goat antimouse IgA-horse radish peroxidase (HRP) conjugate was added and the plates were incubated for 60 min. The HRP substrate tetramethylbenzidine (TMB) was added and the plates were incubated overnight at 4°C. The reaction was terminated with 2 M H2SO4 and absorbance was measured at 450 nm in an automated microplate reader (Microplate Reader model 680, Bio-Rad Labs., CA, USA).

Saliva contains different forms of IgA: IgA monomer, IgA dimer, secretory component, J-chain, and sIgA. The sIgA is a polymeric molecule composed of IgA monomers, J-chain and secretory component, and plays a role in mucosal immunity. In the present study, antibody to mouse IgA was used to measure salivary IgA in ELISA because the purpose of the study was to examine how acute surgical stress influences IgA secretion.

6. Competitive RT-PCR amplification

Total RNA extraction, reverse transcription, and competitive polymerase chain reaction (PCR) amplification proceeded as described. Briefly, total RNA was isolated from individual salivary gland tissues according to the manufacturer’s instructions (FastRNATMKit-GREEN, BIO 101, Vista, CA, USA). The RNA was reverse transcribed to cDNA with 200 units of Superscript II reverse-transcriptase (Life Technologies Inc., Rockville, MD, USA). The IgA primers were 5'-TTC CGT GCA ACA TGA CTC TA-3' and 5'-GGT GAA AGC AGC TCC CTC AG-3'. Internal standards (competitors) were constructed according to the manufacturer’s instructions (Competitor DNA Construction Kit; Takara Biomedicals, Shiga, Japan).

Amplified PCR products were separated by electrophoresis in 3% agarose gels containing ethidium bromide. Fluorescence intensity in bands of the target gene and their respective internal standards were measured using an image analyzer (Molecular Imager FX, Bio-Rad, Hercules, CA, USA) and the quantity of IgA mRNA was calculated by comparison with standard curves. The quantity of IgA was normalized to that of S16 ribosomal protein mRNA.

7. Statistical analysis

All values in the text and figures are expressed as means ± SE. The Mann-Whitney U test was used to statistically analyze the data from the study of surgically stressed mice. The values were compared with those of normal control mice. Changes in IgA secretion in the presence of antagonists underwent multiple comparisons using ANOVA, and then Scheffe’s method was used to compare groups. Significance was established at p < 0.05.

Results

1. Salivary flow rates and protein concentrations in surgically treated mice

We evaluated the effects of surgical insult under general anesthesia upon the flow rate and composition of saliva. Figure 1 shows the flow rates of saliva secreted after the administration of pilocarpine. Under pre-operative conditions (non-surgically treated control mice group), the flow rate of saliva was 0.119 ± 0.014 ml/10 min. Immediately after surgery, the flow rate significantly decreased (0.044 ± 0.007 ml/10 min, p < 0.01). On the first post-surgical day, the flow rate returned to the pre-surgical level (0.101 ± 0.012 ml/10 min). No significant changes were observed in the salivary flow rate at any time during the next 4 weeks. Saliva was collected under general anesthesia in all of the experiments, so that the obtained results were not affected by the anesthesia.

Figure 2 shows that the surgical procedure immediately caused an increase of about 6-fold in the salivary protein concentration (8.107 ± 1.079 mg/ml) when compared with that in control mice (1.283 ± 0.146 mg/
ml, p < 0.01. The concentration did not remain elevated after day 1.

2. Salivary IgA concentrations in surgically treated mice

Surgery significantly affected the IgA concentrations (Fig. 3) as reflected in changes in the protein concentration (Fig. 2). Salivary IgA concentrations in the Surgically treated and preoperative control mice were 13.035 ± 3.555 and 5.868 ± 1.926 µg/ml, respectively (Fig. 3A). The modality of the increase and decline of the IgA concentration (Fig. 3A) was similar to that of protein concentration (Fig. 2). However, the magnitude of the IgA elevation (Fig. 3A) was not as high as that of the protein concentration (Fig. 2). And the total output of IgA during 10 min saliva collection time [IgA concentration (µg/ml) × Saliva flow rate (ml/10min)] was unchanged compared with the non-surgically treated control mice group: 0.464 ± 1.414 and 0.491 ± 1.626 µg/10 min, respectively.

Figure 3B shows the calculated (%) IgA levels as a relative constituent of secreted salivary proteins. The IgA levels in salivary proteins tended to decrease immediately after surgery and increase during the first week,
although the difference was not significant.

3. IgA mRNA in the surgically treated mice

The amount of mRNA did not significantly change after surgical insult (Table 1).

4. Effects of antagonists upon IgA secretion in surgically treated mice

Yohimbine, a $\alpha_2$-receptor antagonist, suppressed the increase of IgA concentration in saliva caused by surgical insult, whereas, prazosin, $\alpha_1$-receptor antagonist, had no effect (Fig. 4A). The increase of concentration induced by surgery was almost completely antagonized by prior exposure to yohimbine. The $\alpha$-adrenergic receptor antagonists did not affect the IgA secretion stimulated by pilocarpine in the control mice.

The levels of protein concentration was not suppressed (rather increased) in the surgically stressed animals pre-treated with prazosin or yohimbine (Fig. 4B), in which the salivary flow rate in the mice pre-treated with antagonists also showed a similar level to that of the surgically treated mice.

Thus, the amount of IgA secretion was significantly decreased in the mice pre-treated with yohimbine: $0.153 \pm 0.030$ and $0.464 \pm 1.141$ mg/10 min, respectively. The results indicate that the IgA increase was mainly evoked by $\alpha_2$-adrenergic receptor simulation under conditions of acute stress.

Discussion

The present study showed that the levels of salivary IgA concentration were significantly elevated immediately after surgery in mice. Although general anesthesia itself increases stress, the transient increase in sali-

**Table 1** IgA mRNA in salivary gland from surgically treated mice.

<table>
<thead>
<tr>
<th></th>
<th>Parotid gland</th>
<th>Submandibular gland</th>
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<tbody>
<tr>
<td>Control</td>
<td>22.48 ± 5.04</td>
<td>28.94 ± 2.46</td>
</tr>
<tr>
<td>Immediately after surgery</td>
<td>26.07 ± 3.74</td>
<td>21.84 ± 3.60</td>
</tr>
<tr>
<td>Days post-surgery 1</td>
<td>14.16 ± 5.46</td>
<td>26.94 ± 6.89</td>
</tr>
<tr>
<td>7</td>
<td>20.11 ± 4.89</td>
<td>26.45 ± 6.77</td>
</tr>
<tr>
<td>14</td>
<td>36.13 ± 5.83</td>
<td>16.45 ± 4.22</td>
</tr>
</tbody>
</table>

Values are means ± SE of parotid and submandibular mRNA from 15 to 17 surgically treated mice. IgA mRNA levels are expressed as IgA/S16 × 1000. Pre- and post-surgical conditions did not significantly differ.
vary protein and salivary IgA concentration and decrease in salivary flow rate were definitely due to surgical stress.

Autonomic nerves supplying the glands regulate the rate of IgA secretion, which is enhanced by electrical stimulation of both parasympathetic and sympathetic nerves. Similarly, Proctor et al. reported that IgA was stimulated by both muscarinic and α- and β-adrenergic agonists. They demonstrated that the most IgA secretion was evoked by the α-adrenergic agonist phenylephrine and reduced by the β-adrenergic antagonist propranolol. Winzer et al. showed that an increase in IgA concentration that was not diminished by a β-adrenergic antagonist propranolol. Winzer et al. showed that an acute stressor elicited an increase in the human sIgA concentration that was not diminished by a β-adrenergic antagonist, suggesting that sIgA is not regulated only by β-adrenergic stimulation. These observations suggest that IgA secretion is stimulated by autonomic nerves, but the mechanisms have remained obscure, and the intensity of stimulation seems to depend on individual causative factors. To clarify the specificity of autonomic receptor stimulation with respect to the increase of IgA in surgically treated mice, we administered mice with an α-adrenergic antagonist. The results demonstrated that the elevated IgA in surgically stressed mice was mediated by α2-receptor stimulation, because yohimbine completely antagonized the surgically induced enhancement of salivary IgA concentration. These findings indicated that IgA secretion is mainly regulated by α2-adrenergic stimulation during acute stress.

During sIgA secretion, dimeric IgA molecules are locally generated by plasma cells that produce IgA in the lamia propria of mucosal membranes or in the connective tissue of glands. The dimers are transcytosed to the apical surface of the epithelial cells after which IgA is released in the secretory form. Competitive RT-PCR for IgA mRNAs in the present study showed that plasma cells from surgically treated mice did not produce IgA over the control mice level. Carpenter et al. reported that the increased secretion of IgA does not depend upon increased plasma cell activation, since isolated plasma cells from the salivary gland did not respond to agonists. On the other hand, they demonstrated that sympathetic nerve stimulation increases the amount of pIgR expressed on the membrane surface, and that stimulating salivary gland cells with adrenaline increases cellular IgA uptake. Thus, stimulation of α2-adrenergic receptors might regulate the transcytosis of pIgR.

Salivary IgA has showed potential as a stress marker in humans and in other animals. Chronic stress, such as during revision periods prior to university examinations, is associated with reductions in sIgA. Contrarily, sIgA transiently increases in response to acute psychological stress such as public speaking, computer game challenges and mental arithmetic. The present data suggested that salivary IgA could indeed be useful as a stress marker because its concentration became significantly elevated during surgery.

The magnitude of salivary IgA elevation immediately after surgery determined here was slightly less than that of the total saliva protein concentration (Fig. 3B). Namely, IgA levels calculated as a relative constituent of proteins in secreted saliva and expressed as a percentage of salivary protein were slightly lower than preoperative values. The total output of IgA during 10 min saliva collection time was unchanged compared with the control mice, suggesting that the impairment of function of mucosal immunity as described in the animal model did not occur.

Other animal experiments have demonstrated that surgical stress enhances the secretion of salivary proteins, mainly through α1-adrenergic receptor stimulation. This is because transient norepinephrine secretion from sympathetic nerve endings stimulates the adrenergic receptors. Norepinephrine binds more potently to α- than to β-adrenergic receptors, which might contribute to the response of salivary gland secretion. Thus, salivary proteins secreted upon α-receptor stimulation should constitute a more appropriate stress marker than those stimulated through β-adrenoceptors. Although further studies are necessary to define the most appropriate candidates, salivary proteins can serve as sensitive and objective markers of stress in humans because samples can be non-invasively obtained.

The results of our study that surgical stress enhances the concentration of salivary IgA, in which the total output of IgA during 10 min was unchanged, contradict the previous report. In surgical models of stress, such as themal injury, femur fracture, hapatectomy, and hemorrhagic shock, a significant decrease in sIgA occurs in a variety of secretions including bile, pulmonary secre-
tions and succus entericus. This effect was speculated to be due to the release of glucocorticoids since the administration of glucocorticoids to rats results in a time- and dose-dependent decrease in mucosal immunity. In the secretion of IgA from the salivary gland in this study, acute surgical stress directly stimulates sympathetic nerves, and stimulation of sympathetic nerves demonstrated the intense and transient increase of IgA concentration. However, the subsequent suppression of IgA secretion did not occur in this experiment, suggesting that the acute stress did not perturb the mucosal immune system.

The IgA in saliva protein tended to increase during the first week after surgery, although the difference was not significant. The elevated IgA might benefit the host as cellular immunity is depressed by the neuroendocrine response to surgery, and mucosal immunity might compensate. Secretory IgA acts similarly to mucin by aggregating bacteria. It also neutralizes bacterial toxins and enzymes and prevents the absorption of allergens as well as carcinogens through mucosal membranes into the body. Immune complexes formed beneath epithelial cells after antigen absorption are transported into secretions by a pIgR dependent transport system. We could not determine such functions in salivary IgA from the present study. A possible mechanism of the prolonged increase in secretion of IgA might be the stimulation of B-cells with adrenergic receptors. The B-cell, precursors of IgA-producing plasma cells, are activated in mucosal associated lymphoid tissue (MALT), and then migrate into the blood circulation until they reach the salivary gland where they remain. Since stimulating B-cells with a β-adrenergic receptor stimulant enhanced both the number of antibody-secreting cells and the total level of antibody produced by B-cells, norepinephrine secreted by surgical stress might enhance the cells in the MALT. Increased production of IgA in salivary tissue might enhance transepithelial transport mediated by pIgR.

In conclusion, it is suggested that surgical stress does not perturb mucosal immunity in our experimental model, although a transient increase of concentration of salivary IgA was observed immediately after surgical insult.

Acknowledgments
The authors thank Professors Katsunori Ishibashi, Ichiro

Saito and Yasutake Saeki for critical reading of the manuscript.

References


