vibration on the SVV and nystagmus on the same day. Seven patients underwent tumor removal via a translabyrinthine approach (six patients) or a retrosigmoid approach (one patient). Three of these seven patients were examined before and after surgery (translabyrinthine approach), and four patients were only examined after surgery (translabyrinthine approach, three patients; retrosigmoid approach, one patient). The average duration between operation and postoperative examination was 8.9 week (2–32 weeks). On the other hand, seven patients had not undergone surgery at the time of examination.

The SVV was measured using custom-built equipment (Nagashima Medical Instruments Co., Ltd., Tokyo, Japan), which corrects the visual vertical automatically at the beginning of the measurement, based on a built-in level. The patient was presented with a light emission diode (LED) bar (130-mm length, 1-mm width) mounted on a monitor board, which was easily rotated in a clockwise or counterclockwise direction using manipulation buttons, and the angle of the bar could be measured at 0.1° interval (Fig. 1). The vibration source was a commercially available, handheld massager (YCM-8; Yamazen, Higashiosaka, Japan) oscillating at 110 Hz. Vibration was applied to the (right and left) dorsal neck muscle area approximately 3–4 cm below the occipital skull.

The SVV was assessed in the sitting position 0.9 m away from the monitor in a dark room, with the head and chin of the patient fixed with a retainer device. The SVV was measured under the following three different conditions: no vibration, and vibration to the right dorsal neck and to the left dorsal neck. Each measurement condition was tested four times successively, and the average value was calculated. At the beginning of the measurements, the initial position of the bar was set alternately in the clockwise and counterclockwise directions, and the patients were asked to align the LED bar along the axis of the SVV. In the present study, the normal range of the SVV was defined as ±3° from the gravitational vertical, based on previous SVV measurements [4].

Spontaneous nystagmus, as well as nystagmus induced by vibration at the right and left dorsal neck, was observed using infrared goggles. If nystagmus was observed under any condition, spontaneous as well as vibration-induced nystagmus was recorded using electronystagmography (ENG), and the slow phase velocity (SPV) of the horizontal component of the nystagmus was assessed. Nystagmus was recorded for about 30 s for each condition and the maximum SPV was calculated.

All parts of the present study were performed in accordance with the guidelines of the Declaration of Helsinki.

Results

Representative SVV and ENG recordings of nystagmus obtained from a female patient with left vestibular schwannoma are presented in Fig. 2. Her tumor was totally removed through the translabyrinthine approach 8 months previously. No apparent nystagmus was observed without neck vibration, but nystagmus directed to the right side was induced by vibration to both the right and left dorsal neck (Fig. 2 left column). Maximum SPV induced by vibration to the left (ipsilateral to the lesion) and the right (contralateral to the lesion) neck was 7.52 and 7.21°/s, respectively. SVV without vibration and with vibration to the left (ipsilateral) and right (contralateral) neck was 2.025°, 4.35° and 3.125°, respectively, indicating that the SVV was slightly shifted to the left (ipsilateral) side without vibration, and was shifted further to the left side by neck vibration. Therefore, the SVV shift was greater if vibration was applied to the ipsilateral neck as previously reported [4]. The shift of the SVV and the SPV of nystagmus were directed to the ipsilateral side in most patients. Therefore,

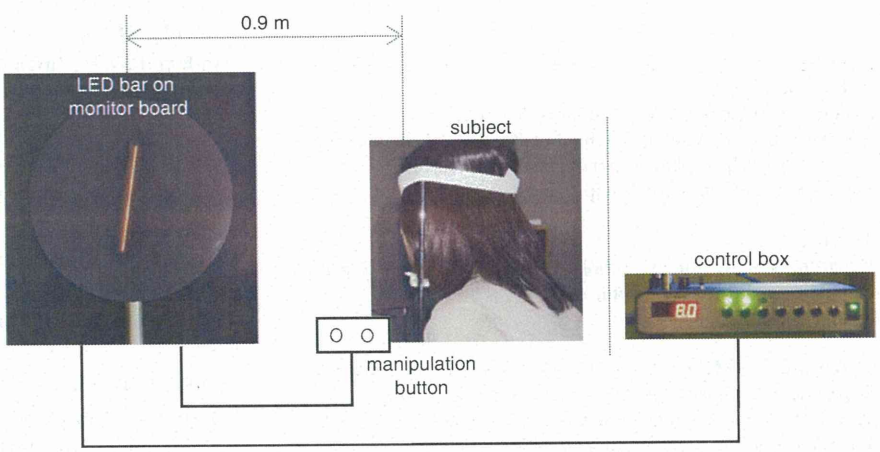

Fig. 1 Schema of SVV measurement. SVV was measured using custom-built equipment (Nagashima Medical Instruments Co., Ltd., Tokyo, Japan) positioned 0.9 m away from a subject. Subjects were asked to adjust the LED bar (130-mm length, 1-mm width) along the axis of the subjective vertical (see text for further details)

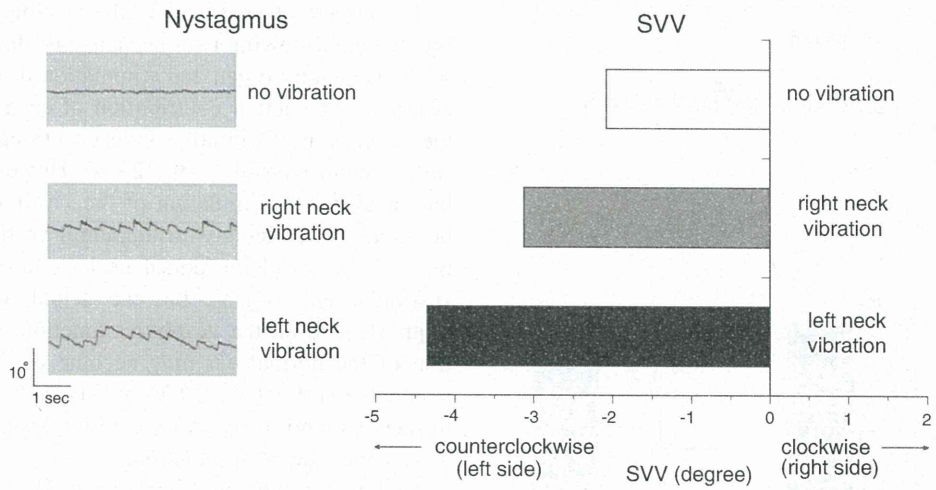

Fig. 2 Typical examples of SVV and ENG recordings of nystagmus obtained from a patient after surgery for left vestibular schwannoma (62 years old, female). ENG recording without vibration, with vibration of the right dorsal neck and with vibration of the left dorsal neck are shown in the *left column*. Vibration of the right and left dorsal neck caused apparent nystagmus directed to the right (intact) side. SVV values without vibration, with vibration of the right dorsal neck and with vibration of the left dorsal neck are presented in the *right column*. SVV was slightly shifted to the left (pathological) side without vibration, and was shifted more to the left side by neck vibration

in the following analysis, the direction of SVV and SPV of nystagmus were given positive and negative values to indicate the ipsilateral and contralateral sides of the lesion, respectively.

The relationship between the SVV value and SPV of the nystagmus is presented in Fig. 3. Nystagmus occurred in 79% of cases of conditions with the SVV shifted by more than 3° from the gravitational vertical, and in only 22% of cases with shift of the SVV within 3°. Figure 3 indicates a significant link between SVV and SPV of the nystagmus ($r = 0.452$, $P < 0.01$). However, analysis of the correlation in the selected cases with nystagmus found no correlation between SPV and SVV. The shift of the SVV and the SPV of the nystagmus were directed to the ipsilateral side of the lesion, except in one preoperative patient in whom the SVV shifted to the contralateral side by more than 3° without nystagmus.

Figure 4 compares the positive rate of abnormal SVV (SVV shifted by more than 3° from the gravitational vertical) with the presence of nystagmus. The positive rates of abnormal SVV and the presence of nystagmus under the three different conditions (no vibration, ipsilateral vibration and contralateral vibration) were very similar (no significant differences). Both significantly increased from about 20 to 60% with vibration ($P < 0.05$).

Figure 5 shows the effects of neck vibration on the SVV and SPV of nystagmus. Mean shift of SVV and mean SPV of nystagmus were significantly increased by neck vibration ($P < 0.05$), with significantly larger effects on the ipsilateral compared to the

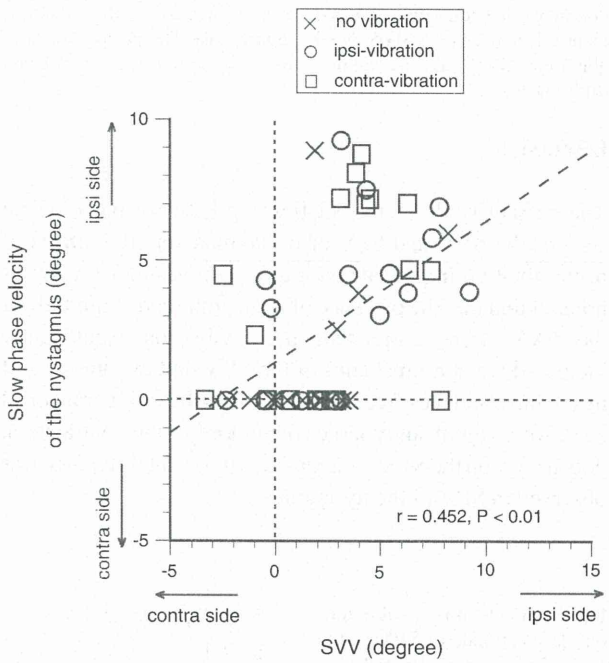

Fig. 3 Relationship between magnitude of SVV and maximum SPV of nystagmus. The magnitude of SPV of the nystagmus was plotted as a function of shifts of SVV. The directions of SVV and nystagmus were determined based on the pathological side (positive and negative values indicate that shift of SVV and SPV of nystagmus were directed to the ipsilateral and contralateral sides of the lesion, respectively) (see text for further details)

contralateral side for SVV ($P < 0.05$), but no significant difference between the ipsilateral and contralateral sides for SPV.

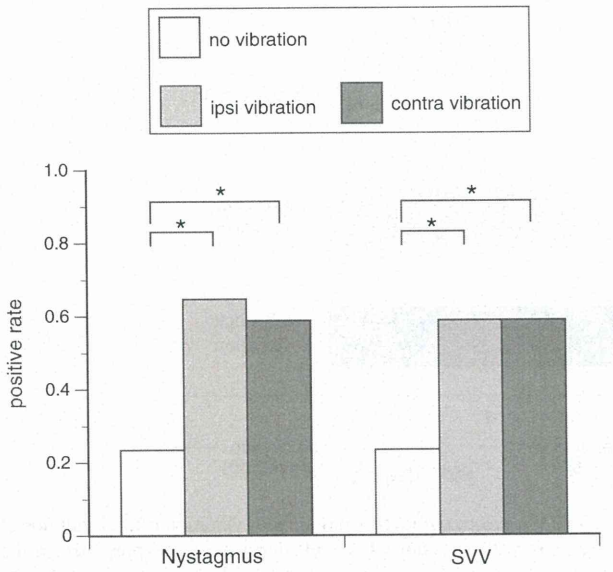

Fig. 4 Increased detectability of SVV and nystagmus by neck vibration. The positive rate of abnormal SVV (SVV shifted more than 3° from the gravitational vertical) and nystagmus was divided into three different conditions (no vibration, and ipsilateral and contralateral neck vibration). *Asterisks* in the figure indicate significant differences ($P < 0.05$ by Chi square test). The positive rates of abnormal SVV and nystagmus are very similar (no significant differences)

Discussion

The present study of the relationship between pathological shift of the SVV and SPV of nystagmus, and the effects of neck vibration in patients with unilateral vestibular schwannoma found that the presence of nystagmus and magnitude of the SVV were correlated, neck vibration significantly increased the abnormal shift of the SVV and the presence of nystagmus, and the effects of vibration to the ipsilateral dorsal neck were significantly larger than those to the contralateral dorsal neck on the SVV, whereas no significant difference was observed in SPV of the nystagmus.

The effects of vibration on the vestibular system may involve the following two mechanisms: direct stimulation of the vestibular organ and stimulation of muscle spindle afferents. The actual contribution of each mechanism to the generation of vibration-induced nystagmus and SVV shift is controversial [4, 9, 12–14]. However, the contribution of direct stimulation of the vestibular organ may be greater in patients with unilateral vestibular dysfunction. Vibration of the dorsal neck could affect bilateral vestibular end organs, but the actual activation level might depend on the vestibular function, as the stimulation of the normal ear may become predominant. As a result, the shift of the SVV and the SPV of the evoked nystagmus tend to be directed to the lesion side, regardless of the side of stimulation.

Vibration is known to cause the SVV to shift to the stimulated side in normal subjects, and the stimulation of muscle spindle afferents may be mainly responsible for this phenomenon [4, 7, 12, 15]. Thus, if SVV shift reflects the summed effects of direct stimulation of the vestibular organ and neck muscle spindle, the predominant effect of ipsilateral stimulation on the SVV, as seen in the present as well as several previous studies [4, 12, 13], may be explained as ipsilateral shift of SVV caused by vestibular unbalance that is enhanced by ipsilateral neck stimulation, but diminished by contralateral neck vibration. In contrast, such differences with the side of neck vibration were not observed in nystagmus in patients with unilateral vestibular dysfunction [9, 11]. Why such differences cannot be observed in nystagmus, but are observed in the SVV, remains unclear, although the relative magnitude of vibration effects via muscle spindle afferents and direct stimulation of the vestibular end organ might be different.

The present study indicates that both SVV and nystagmus affected by neck vibration have many similar clinical features, and may be important in assessing unilateral vestibular dysfunction.

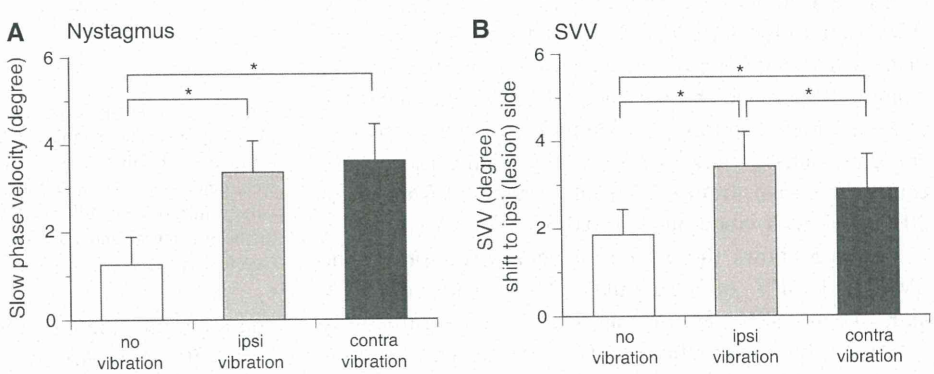

Fig. 5 Effects of neck vibration on the magnitude of SVV and SPV of nystagmus. *Asterisks* in the figure indicate significant differences ($P < 0.05$ by paired t test) (see text for further details)

Conflict of interest The authors declare that they have no conflict of interest.

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Tohoku J. Exp. Med., 2011, 223, 17-25

Surgical Treatment Is Recommended for Advanced Oral Squamous Cell Carcinoma

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Oral squamous cell carcinoma is one of the most frequent types of head and neck cancers in Japan. Although recent reports have shown positive results of non-surgical treatment for advanced head and neck squamous cell carcinoma, including tongue cancer, no clear treatment strategies have been established for oral cancers, except for tongue cancer. To assess appropriate therapies, we conducted a retrospective chart review of 114 Japanese patients with oral cancers that were pathologically diagnosed as squamous cell carcinoma, excluding tongue cancers. The overall and the disease specific 5-year survival rates were 53% and 61%, respectively. Univariate and multivariate analyses revealed a lower stage (I, II, or III) and non-surgical treatment as good and poor prognostic factors of oral squamous cell carcinoma, respectively, based on their hazard ratios of 0.17 (95% CI 0.045-0.60, $p = 0.0061$) and 5.3 (95% CI 2.7-11, $p < 0.0001$). Furthermore, impact of surgery was well documented in the operable stage IVa cancers ($p = 0.00015$). The surgical treatment consisted of the wide resection of the primary tumor and the neck dissection for stage III or IV tumors. The present data also suggest that adjunctive therapy, such as post-operative radiation therapy or post-operative chemo-radiation therapy, shows no survival benefit compared to the surgery alone. We therefore recommend the surgical treatment for advanced oral squamous cell carcinoma in Japanese patients. These results would be helpful in future clinical trials, especially in non-surgical treatment studies of oral squamous cell carcinoma in Japan.

Keywords: oral cancer; buccal mucosa; gingiva; oral floor; hard palate
Tohoku J. Exp. Med., 2011, **223** (1), 17-25.

In Japan, oral cancer is one of the most frequent types of head and neck cancers (Matsuda et al. 2009). We have previously reported on prognostic factors and treatment strategies for tongue squamous cell carcinoma (tongue SCC) that is the most common type of oral cancers (Tateda et al. 2000; Shiga et al. 2007).

Recent research revealed that chemo-radiation therapy (CRT) seems to be effective for advanced head and neck squamous cell carcinomas (head and neck SCC) including oral squamous cell carcinomas (oral SCC) (Robbins et al. 2005; Doweck et al. 2008; Fuwa et al. 2008; Stenson et al. 2010). However, the treatment strategies have not yet been established for oral SCC, excluding tongue SCC, such as SCC in buccal mucosa, gingiva, hard palate and oral floor, because of the rare incidence (Ariyoshi et al. 2008). Actually, major clinical studies about CRT for oral SCC included a large number of tongue SCC and a small number of other oral SCC. Referring to previous reports regarding poor prognostic factors for oral SCC excluding tongue SCC (Soo et al. 1988; Fang et al. 1997; Dias et al. 2003), T4a and N2b in accordance with the guidelines of the Union Internationale Contre le Cancer (UICC) 2002 have been considered to be prognostic factors; however, the available data are still insufficient to draw any significant conclusions or select the optimal treatments for oral SCC.

We herein report a retrospective analysis of the treatment outcomes of patients with oral SCC other than tongue SCC to develop appropriate treatment strategies at every stage. This study was performed at two facilities that have the same treatment policy, basically consisting of a wide resection or intensive CRT.

Subjects and Methods

The subjects included 114 patients with oral SCC (excluding tongue SCC) who received inpatient treatment at the Department of Otolaryngology-Head and Neck Surgery of the Tohoku University School of Medicine and the Division of Head and Neck Surgery of the Miyagi Cancer Center between January 1993 and December 2004.

Received March 11, 2010; revision accepted for publication December 3, 2010. doi: 10.1620/tjem.223.17
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Institutional review board approval was obtained for this study, and written informed consent was obtained from all patients. Before treatment, staging was conducted for all patients using tumor biopsy, head and neck CT, head and neck MRI, thoracoabdominal CT, bone scintigram, etc. In the diagnosis of cervical lymph node metastases with CT and MRI, submandibular lymphadenopathy of 15 mm or more, those in other sites of 10 mm or more, and those with central necrosis were considered positive. Staging was conducted in accordance with the guidelines of the UICC 2002. Follow-up periods were calculated from the day when the initial treatment had been started. The median follow-up period was 44 months (range 1-146 months), and the follow-up period of all surviving patients was 20 months or more. All follow-up data were updated at the end of October 2008.

In the classification by sites, hard palate SCC and upper gingival SCC were analyzed in combination due to the anatomical continuity and the small number of patients. The treatment policy at both of these facilities was basically surgery. Regarding the postoperative adjunctive treatment, post-operative radiation therapy (PORT) or post-operative chemo-radiation therapy (POCRT) for patients with cancer-positive stumps, multiple cervical lymph node metastases and extracapsular progression of lymph node metastasis were performed unless the patients rejected them. The surgical treatment basically consisted of a wide resection of the primary tumor and a neck dissection for stage III or IV tumors. In addition, for patients, in whom surgery could not be performed due to patient refusal, definitive treatment, mainly with definitive RT or CRT (RT/CRT) was thus conducted. Furthermore, RT/CRT was basically conducted for inoperative patients with T4b, M1 or other prominent disorders including multiple primary cancers. Those patients received the standard fractionated radiation therapy given at a dosage of 4-MV X-rays of 2 Gray (Gy) per fraction, 5 fractions per week.

Regarding the CRT regimen, platinum agents, such as cisplatin (CDDP), and/or fluorouracil (5FU) were concomitantly administered to the patients. Only one patient received docetaxel additionally. Our standard regimen of CRT is as follows: iaCDDP, intra-arterial infusion of high dose CDDP (100-150 mg/m^2 weekly, 4-6 times); CF, systematic infusion of CDDP (70 mg/m^2 day 2) and 5FU (1,000 mg/body day 1-5) every four weeks, 2 times; ivCDDP, systematic infusion of CDDP (50 mg/m^2) weekly, 4-6 times; and DCF, systematic infusion of combined CDDP (60 mg/m^2 day2), 5FU (1,000 mg/body day 1-5) and docetaxel (50 mg/m^2 day 2) every four weeks, 2 times.

The patients were analyzed for gender, age, TNM classification, stage, site and initial therapeutic regimen, recurrence, period before recurrence and survival. Survival rates were calculated using the Kaplan-Meier method, and significant differences were analyzed with the log-rank test. Univariate and multivariate analyses were conducted using the Cox proportional-hazards model. For the analyses, Stat View version 5.0 for Windows (SAS Institute Inc, Cary, NC) was used. The statistically significant level was 0.05.

Results

Clinical features of the patients with oral SCC

The patients included 85 males and 29 females, aged from 40 to 89 years (median age: 69 years). In the T classification, T4 cancers accounted for 43% of the patients, and in the N classification, lymph node metastases accounted for 50% and N2 patients for 31%. In the classification by stage, advanced cancer (stage III or IV) accounted for 68% of the patients, of which stage IV cancer accounted for 55%. In the classification by site, the buccal mucosa was the most common site and was involved in 37 patients, followed by the oral floor in 35 patients, the lower gingiva in 28 patients, and the upper gingival or hard palate in 14 patients. For the initial therapeutic regimen, 92 patients (81%) underwent treatment including surgery, and 22 (19%) patients did not undergo surgery. The reasons for performing non-surgical treatment were due to simple rejection (4 patients), organ preservation (4 patients), advanced age (4 patients), inoperable stage (T4b or M1) (5 patients), and other disorders including multiple primary cancers (5 patients). Adjunctive therapy for the surgical treatment included PORT for 22 patients, POCRT for 1 patient, and pre-operative or post-operative chemotherapy (6 patients). The reason why POCRT was chosen was due to the fact that the patient had synchronous multiple primary cancers, *i.e.*, advanced cancer of oral floor and esophageal cancer. He was treated with POCRT for oral cancer and with definitive CRT for esophageal cancer. On the other hand, the choices of adjunctive chemotherapy were due to neo-adjuvant chemotherapy prior to surgery in 3 patients, distant metastasis found before surgery in 1 patient and adjunctive chemotherapy protocol until 1998 in 2 patients. The post-operative radiation dose ranged from 46 to 70 Gy (median: 50 Gy) in the patients treated.

Survival analysis

Regarding the survival analysis using the Kaplan-Meier method, the 3-year and 5-year overall survival rates were 59% and 53%, respectively. The 3-year and 5-year disease-specific survival rates were 65% and 61%, respectively.

When the 5-year disease-specific survival rate was analyzed by factor, patients with T4a (43%) or T4b (0%) showed significantly poor prognoses in the T factors, and patients with N2b (48%) or N2c (19%) showed significantly poor prognoses in the N factors (Table 1). In addition, according to stage, stage IV cancers, which accounted for the majority of the patients, had poor prognoses (Fig. 1). In the classification by site, patients with buccal mucosal cancer (47%) tended to show poor prognoses, but no statistical significance was observed (Table 1). In the classification by the initial therapeutic regimen, 92 patients, in whom the treatment was mainly surgery, had a 5-year disease-specific survival rate of 71%, while the 5-year disease-specific survival rate of 22 other patients with non-surgical treatment was 20%, indicating a significantly poor prognosis of non-surgical treatment ($p < 0.0001$) (Fig. 2A).

Regarding adjunctive therapy in combination with the surgical treatment, the 5-year disease-specific survival rates of surgery alone ($n = 63$), PORT ($n = 22$), POCRT ($n = 1$) and pre-operative or post-operative chemotherapy ($n = 6$) were 77%, 53%, 0% and 75%, respectively. Furthermore, when only stage IV patients were examined, these rates of surgery alone ($n = 25$), PORT ($n = 18$) and pre-operative or

Table 1. Disease-specific survival rates according to patient profiles and tumor characteristics.

Factors	Category	No. of Patients	5-year survival (%)	*p* value
Gender	<1> Male	85	58	$p = 0.54$
	<2> Female	29	68	
Age	<1> ≤ 65	45	57	$p = 0.94$
	<2> > 65	69	63	
T	<1> T1	13	77	2 vs 4 $p = 0.0013$
	<2> T2	39	78	1 vs 5 $p = 0.00012$
	<3> T3	13	67	2 vs 5 $p < 0.00001$
	<4> T4a	46	43	3 vs 5 $p = 0.0056$
	<5> T4b	3	0	4 vs 5 $p = 0.015$
N	<1> N0	57	80	1 vs 2 $p = 0.032$
	<2> N1	21	54	1 vs 3 $p = 0.028$
	<3> N2a	4	25	1 vs 4 $p = 0.019$
	<4> N2b	20	48	1 vs 5 $p < 0.00001$
	<5> N2c	12	19	
Stage	<1> I	9	100	1 vs 4 $p = 0.011$
	<2> II	27	84	1 vs 5 $p = 0.00019$
	<3> III	15	79	1 vs 6 $p = 0.0025$
	<4> IVa	56	43	2 vs 4 $p = 0.0023$
	<5> IVb	3	0	2 vs 5 $p < 0.00001$
	<6> IVc	4	-	2 vs 6 $p = 0.00027$
				3 vs 4 $p = 0.043$
				3 vs 5 $p = 0.0013$
				3 vs 6 $p = 0.010$
				4 vs 5 $p = 0.0015$
Site	<1> Buccal mucosa	37	47	1 vs 3 $p = 0.19$
	<2> Oral floor	35	68	
	<3> Lower gingiva	28	69	
	<4> Upper gingiva Hard palate	14	57	
Treatment	<1> Ope	92	71	1 vs 2 $p < 0.00001$
	<1>_ 1 Ope only	63	77	
	<1>_ 2 PORT	22	53	
	<1>_ 3 POCRT	1	0	
	<1>_ 4 Ope+chemo	6	75	
	<2> RT/CRT	22	20	

Ope, Surgery. RT/CRT, Radiation therapy or chemo-radiation therapy.

post-operative chemotherapy ($n = 5$) were 51%, 47% and 75%, respectively. These data of adjunctive therapy have shown no survival benefit compared to surgery alone. In the analysis of significant differences with the log-rank method, the 4 items of T factors, N factors, stages, and initial therapeutic regimens showed statistically significant differences (Table 1).

Univariate analysis

The results of univariate analysis of the disease-specific survival rates are shown in Table 2. Based on the number of patients and the results of the log-rank method, the parameters to be examined included gender (M vs F), age (≤ 65 vs > 65), T (1, 2 or 3 vs 4), N (0 or 1 vs 2), stage (I, II or III vs IV), site (buccal mucosa vs other sites), and treatment method (RT/CRT vs surgery (OPE)). The 4 items of T, N, stage and treatment method showed statistically significant differences in the disease-specific survival rates, and the hazard ratios were 0.32 ($p = 0.0003$), 0.37 ($p = 0.0013$), 0.20 ($p < 0.0001$), and 4.4 ($p < 0.0001$), respectively.

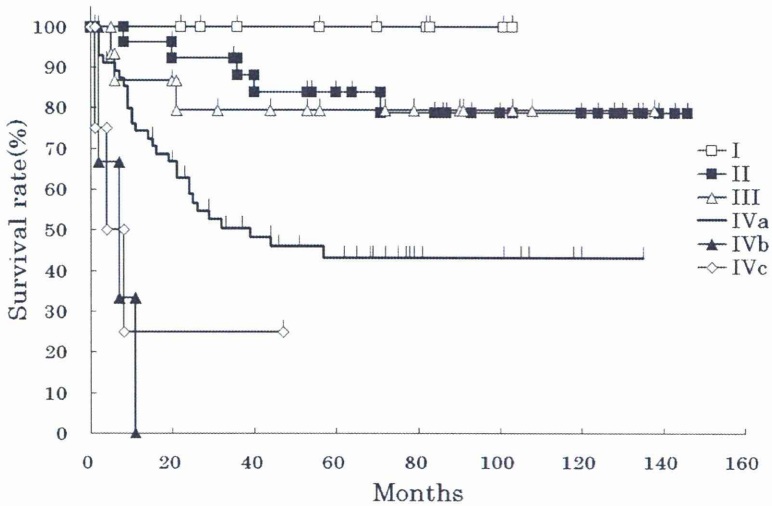

Fig. 1. Disease-specific survival rates by stage.
There was a statistically significant difference as shown in Table 1.

Table 2. Univariate analysis of disease-specific survival rates (Cox proportional-hazards model).

Factors	Category	Hazard ratio	95% CI	*p* value
Gender	M vs F	0.80	0.40 - 1.6	$p = 0.54$
Age	≤ 65 vs > 65	0.98	0.53 - 1.8	$p = 0.94$
T	1 or 2 or 3 vs 4	0.32	0.17 - 0.59	$p = 0.0003$
N	0 or 1 vs 2	0.37	0.21 - 0.68	$p = 0.0013$
Stage	I or II or III vs IV	0.20	0.091 - 0.43	$p < 0.0001$
Site	Buccal mucosa vs Other	1.7	0.90 - 3.0	$p = 0.10$
Treatment	RT/CRT vs OPE	4.4	2.3 - 8.1	$p < 0.0001$

CI, Confidence interval.

Table 3. Multivariate analysis of disease-specific survival rates (Cox proportional-hazards model).

Factors	Category	Hazard ratio	95% CI	*p* value
T	1 or 2 or 3 vs 4	1.3	0.52 - 3.3	$p = 0.57$
N	0 or 1 vs 2	0.81	0.38 - 1.7	$p = 0.60$
Stage	I or II or III vs IV	0.17	0.045 - 0.60	$p = 0.0061$
Treatment	RT/CRT vs OPE	5.3	2.7 - 11	$p < 0.0001$

Multivariate analysis

A multivariate analysis was also conducted for these 4 parameters (T classifications, N classifications, stage and treatment method), and the results are shown in Table 3. Stages I, II and III had a hazard ratio of 0.17 (95% CI 0.045-0.60, $p = 0.0061$) compared to stage IV, and for the treatment method, RT/CRT had a hazard ratio of 5.3 (95% CI 2.7-11, $p < 0.0001$) compared to OPE, which were statistically significant differences. These results were similar to the overall survival rate (data not shown).

Analysis of RT/CRT patients with stage IV

Next, we examined the treatment outcomes of the stage IV and stage IVa patients (excluding inoperable patients). The 5-year disease-specific survival rates of the OPE group were 52% ($n = 48$) and 52% ($n = 46$), for stage IV and IVa, respectively, while those of the RT/CRT group were 0% ($n = 15$) and 0% ($n = 10$), respectively. The RT/CRT group also had significantly poor prognoses compared to the OPE group comparing the stage IV and IVa patients ($p < 0.0001$, $p = 0.00015$, respectively) (Fig. 2B, C). In the patients with stage IVb and IVc, 5 patients who were treated by RT/CRT died within 12 months. On the other hand, 2 patients were treated by OPE. One patient treated by surgery followed by chemotherapy was alive for 47 months after the treatment with disease and the other patient

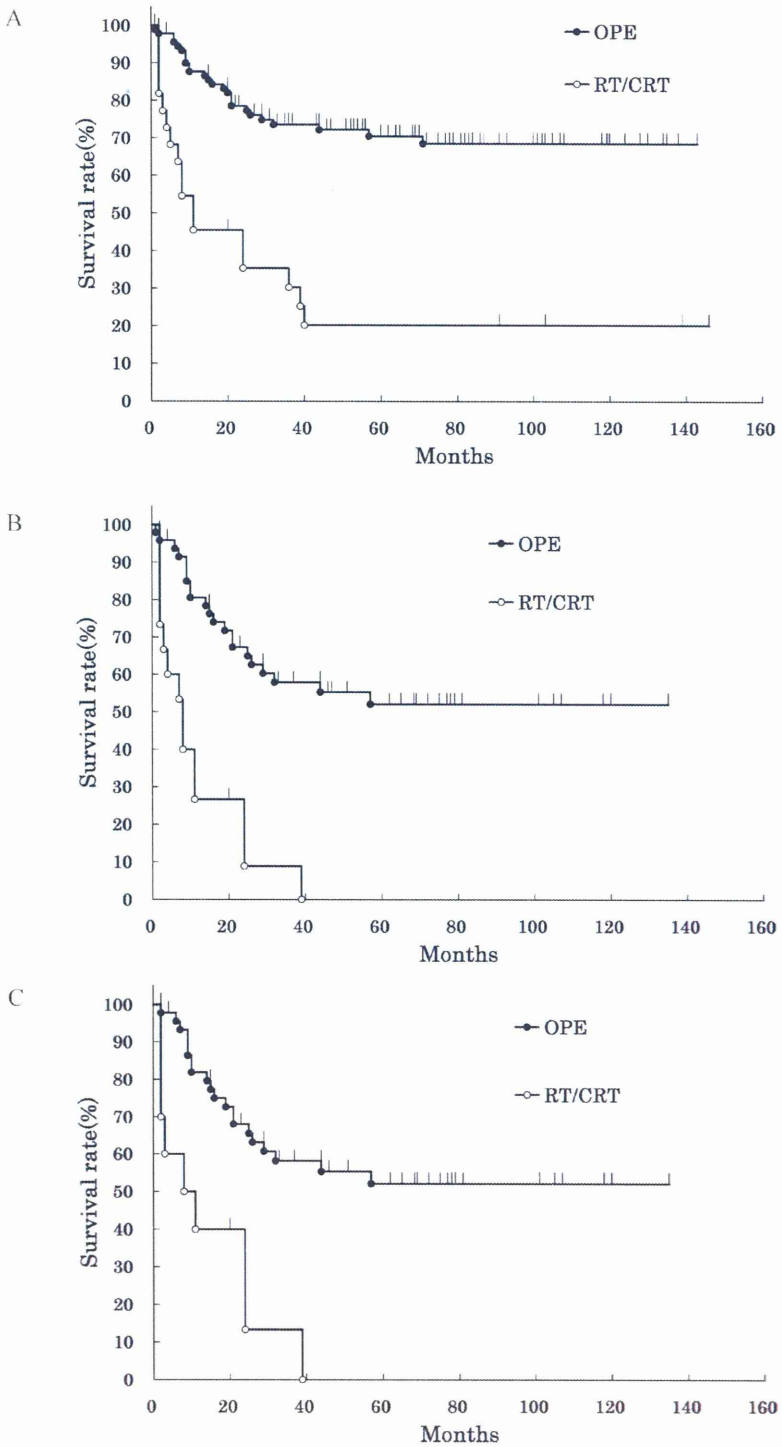

Fig. 2. Disease-specific survival rates by treatment methods.
(A): All patients, (B): Stage IV and (C): Stage IVa
The 5-year disease-specific survival rate of the OPE group showed a significantly good prognosis compared with the RT/CRT group ($p < 0.0001$). Similarly, significant differences were demonstrated in stage IV and IVa patients ($p < 0.0001$, $p = 0.00015$).

Table 4. Disease-specific survival rates for RT/CRT patients.

Factors	Category	No. of Patients	5-year survival (%)	p value
Stage	<1> I or II	5	60	1 vs 3 $p = 0.0053$
	<2> III	2	50	
	<3> IV	15	0	
Site	<1> Buccal mucosa	13	18	1 vs 4 $p = 0.76$
	<2> Oral floor	3	33	
	<3> Lower gingiva	2	0	
	<4> Upper gingiva Hard palate	4	25	
Radiation dose	<1> < 60 Gy	5	0	1 vs 2 $p = 0.029$
	<2> = 60 Gy	5	40	
	<3> 60 Gy <	12	21	
Chemo therapy	<1> With	13	19	$p = 0.70$
	<2> Without	9	22	

treated by surgery alone died 1 month after surgery.

Prognostic factors of the RT/CRT patients

The results of analysis of survival rates by factor of the RT/CRT patients are shown in Table 4. In the classification by stage and by radiation dose, a stage IV (0%) and a radiation dose of less than 60 Gy (0%) had significantly poor prognoses, and there were no significant differences related to the site or the presence or absence of chemotherapy. Five patients received a radiation dose of less than 60 Gy, due to the cessation of treatment because of mucositis or other systematic complications in 4 patients and because of a CRT protocol prior to 1999 in 1 patient. Five other patients received a radiation dose of 60 Gy, according to the radiation therapy protocol against early stage cancers or the CRT protocol against advanced stage cancers prior to 1999.

Among the RT/CRT patients with stage IV disease, 11 patients received CRT, while 4 patients received RT only without chemotherapy (RT only). The chemotherapy regimen consisted of iaCDDP in 4 patients, CF in 4 patients, ivCDDP in 1 patient, DCF in 1 patient, and systematic infusion of only fluorouracil in 1 patient due to renal dysfunction. The radiation doses received by CRT patients were over 60 Gy in 8 patients, and 60 Gy or less in 3 patients. Regarding the clinical efficacy of CRT, 6 of the 11 patients treated by CRT demonstrated a complete response of the local tumor in the irradiated volume at the end of the primary treatment, although recurrent tumors were observed in 4 of these 6 patients at the primary site. A regional recurrent tumor was observed in 1 patient and another patient died of colon cancer 20 months after the initial treatment. No specific correlations between clinical efficacy and CRT protocol were found. On the other hand, all 4 patients treated by RT only demonstrated a partial response or no change. Five patients treated by CRT (3 patients) or RT only (2 patients) died (of pneumonia in 4 patients and of a hemorrhagic gastric ulcer in 1 patient) during the initial treatment.

Regarding the clinical efficacy of RT/CRT in patients with stage I, II or III, the local tumors of all 5 patients with stage I or II tumors demonstrated a complete response. Four patients received RT, while the other patient received CRT. Furthermore, 2 of the 4 patients treated by RT only received irradiation therapy consisting of 60 Gy. Primary recurrent tumors were observed in 2 patients treated by RT only who had received radiation doses of 60 Gy and 64.4 Gy. In stage III, one patient treated by CRT had a complete response, while the others treated by RT only demonstrated a partial response.

Analyses of each site

Regarding the various sites, the 5-year disease-specific survival rates according to the stage and treatment method are shown in Table 5. The same tendencies that stage IV and the non-surgical treatment were significant factors for poor prognoses were observed. The reason why the 5-year disease-specific survival rate of buccal mucosa was the worst probably reflects the fact that a large population of RT/CRT treatment patients were included in buccal mucosa compared to other sites.

Discussion

In this study, we investigated the clinical findings of 114 patients with oral SCC excluding tongue SCC, and our analyses led to the conclusion that poor prognostic factors include stage IV disease and the non-surgical treatment. Although the results of the outcomes in this study support the findings of previous reports (Shaha et al. 1984; Hicks et al. 1997; Sessions et al. 2000), we emphasize that the patient outcome for surgery in our patients was almost equal to, or better than those of previous reports (Hicks et al. 1997; Sessions et al. 2000; Diaz et al. 2003) and this study contained some intensive CRT patients, such as iaCDDP (Doweck et al. 2008), CF (Giralt et al. 2000), and

Table 5. Five-year disease-specific survival rates according to stage and treatment strategy in each site.

Buccal mucosa

Factors	Category	No. of Patients	5-year survival (%)	p value
Stage	<1> I	1	100	2 vs 5 $p = 0.00022$
	<2> II	11	61.4	2 vs 6 $p = 0.0014$
	<3> III	6	83.3	3 vs 5 $p = 0.033$
	<4> IVa	16	31.3	3 vs 6 $p = 0.019$
	<5> IVb	2	0	4 vs 5 $p = 0.0044$
	<6> IVc	1	0	4 vs 6 $p = 0.011$
Treatment	<1> Ope	24	64.5	1 vs 2 $p = 0.0057$
	<2> RT/CRT	13	18	

Oral floor

Factors	Category	No. of Patients	5-year survival (%)	p value
Stage	<1> I	6	100	1 vs 4 $p = 0.027$
	<2> II	8	100	1 vs 6 $p = 0.014$
	<3> III	5	83.3	2 vs 4 $p = 0.019$
	<4> IVa	14	34.4	2 vs 6 $p = 0.0082$
	<5> IVb	0		3 vs 6 $p = 0.019$
	<6> IVc	1	0	4 vs 6 $p = 0.022$
Treatment	<1> Ope	32	71.3	1 vs 2 $p = 0.021$
	<2> RT/CRT	3	33.3	

Lower gingiva

Factors	Category	No. of Patients	5-year survival (%)	p value
Stage	<1> I	1	100	4 vs 6 $p = 0.021$
	<2> II	6	75	
	<3> III	0	83.3	
	<4> IVa	20	56.6	
	<5> IVb	0		
	<6> IVc	1	*	
Treatment	<1> Ope	26	75.9	1 vs 2 $p = 0.022$
	<2> RT/CRT	2	0	

Upper gingiva & Hard palate

Factors	Category	No. of Patients	5-year survival (%)	p value
Stage	<1> I	1	100	4 vs 6 $p = 0.021$
	<2> II	2	100	
	<3> III	3	66.7	
	<4> IVa	6	50	
	<5> IVb	1	0	
	<6> IVc	1	0	
Treatment	<1> Ope	10	70	1 vs 2 $p = 0.14$
	<2> RT/CRT	4	25	

*Alive with disease for 47 months.

DCF (Tsukuda et al. 2010).

Regarding the RT/CRT treatment outcomes in this study, the stage IV patients who underwent RT/CRT had poor outcomes. Although a total of 11 stage IV patients received concomitant CRT, only 2 patients showed tumor-free local control after undergoing concomitant CRT. Moreover, one of these 2 patients showed regional recurrence, and only one stage IV patient could demonstrate loco-regional control after CRT. Although some previous reports have described the effectiveness of RT/CRT for advanced head and neck SCC (Doweck et al. 2008; Stenson et al. 2010), non-surgical treatment for advanced oral SCC has reported not to improve prognosis in Japanese Patients (Inagi et al. 2002; Umeda et al. 2004). These results therefore allow us to consider that advanced oral SCC, excluding tongue SCC, might have a worse response to CRT than other head and neck SCC, and careful examination for any local recurrence should be regularly performed even if a complete remission was obtained, because of their high rates of local recurrence after CRT.

Another problem related to RT/CRT indicated in this study was the occurrence of severe complications, such as pneumonia or hemorrhagic gastric ulcers, resulted in poorer prognoses as well as more aggressive features of the tumors. The reasons for such severe complications are as follows. In this study, 4 patients with an advanced age over 80 years were included in the RT/CRT group, and therefore the adaptive rigidification of the RT/CRT protocol and extreme care when performing RT/CRT appear to be necessary to avoid complications and to reduce the number of deaths due to RT/CRT. Second, this study revealed pneumonia to be the most common complication that could cause a worsening of the outcome for elderly patients. Although dysphagia during or after CRT is a well-known issue (Nguyen et al. 2004), appropriate supportive care, such as dental brushing, might be needed for CRT patients as well as for surgical patients (Akutsu et al. 2010).

Since our results of surgical treatment were considered to be acceptable, these results are thought to be adequate baseline data of future clinical studies. These surgical results were obtained based on wide resection of primary tumors, i.e. a 1.5 cm tumor free margin or negative stump in frozen sections. However, as reported recently (Ota et al. 2009), surgical strategies, such as the resection field and neck dissention, should be investigated in future studies.

Post-operative treatment, such as PORT or adjuvant chemotherapy, did not contribute to any improvement in the survival of our patients, and it only represented a relatively

small number of patients. Considering the results of CRT and adjunctive therapy, these results indicate that sensitivity to radiation and to anticancer drugs might be low in oral cancers, excluding tongue cancer, among Japanese patients. The performance of POCRT for the high-risk group has been recommended (Cooper et al. 2004; Bernier et al. 2004) and PORT alone for the moderate risk group according to NCCN Clinical Practice Guidelines for head and neck cancers, but the utility of post-operative treatment in oral cancer remains controversial (Sadeghi et al. 1986; Inagi et al. 2002; Brown et al. 2007). Our results also indicate that a curative operation with a wide resection of the primary tumor may play an important role in achieving prognostic improvements.

In the past, although some studies have reported that the control rate of localized early cancer with radiation is high (Nair et al. 1988; Bachaud et al. 1994; Yorozu et al. 2001), others have reported that the outcomes of surgical treatment surpass those of RT (Cady and Catlin 1969). At the moment, RT is indicated for small primary cancers for the purpose of local control, and it is believed that surgical treatment is the standard for progressive cancer. In addition, based on this analysis, a radiation dose of 60 Gy or more seems to be required for RT/CRT.

Moreover, although there was no survival benefit of RT/CRT for unresectable patients in this limited study, only 1 patient with stage IVc cancer underwent surgery followed by chemotherapy and remained alive with the disease for 47 months. Although anticancer immuno-chemotherapy using cetuximab (Vermorken et al. 2008) and CRT combined with simultaneous CDDP (Adelstein et al. 2003) are now recognized as standard treatments for metastatic or unresectable head and neck SCC, further studies are needed to develop effective strategies for inoperable patients with oral SCC including surgery and tumor dormant chemotherapy.

Conclusions

Our results suggest that a prolonged survival might be expected if surgical treatment is tolerable for patients with advanced oral cancer. In addition, adjunct treatment did not contribute to any improvement in survival in our study. Since this is a limited retrospective study, a prospective study is therefore considered necessary to prove our hypothesis in the future.

Acknowledgments

This study was supported by a Health and Labour Sciences Research Grant for Clinical Cancer Research (H21-tokubetsushitei-hann-2) from the Ministry of Health, Labour and Welfare, Tokyo, Japan.

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Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2012;157:194–201
DOI: 10.1159/000327545

Received: September 18, 2010
Accepted after revision: March 2, 2011
Published online: October 7, 2011

Induction of Thymic Stromal Lymphopoietin Production by Xylene and Exacerbation of Picryl Chloride-Induced Allergic Inflammation in Mice

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Key Words

Allergic inflammation · Thymic stromal lymphopoietin · Xylene

Abstract

Background: Some chemical compounds in the environment worsen allergic inflammation. In this study, we examined whether organic solvents induce the production of thymic stromal lymphopoietin (TSLP) which elicits Th2-type immune responses. ***Methods:*** Organic solvents were painted on the earlobes of BALB/c mice. The expression of TSLP in the ear was determined by ELISA. ***Results:*** Xylene and toluene, but not chloroform or ethyl acetate, induced the expression of mRNA for TSLP in the earlobe tissue. Among the aromatic compounds, xylene, especially *m*-xylene, and trimethylbenzene caused apparent TSLP production. The level of TSLP in the xylene-treated earlobes reached a maximum at 24 h, and TSLP was expressed in epithelial tissues. Production of TSLP was unaffected in mast cell-deficient W/W^v mice but apparently diminished in TNF-α knockout mice and IL-4 receptor knockout mice. Repeated painting of xylene for 7 days induced an increase in the weight of cervical lymph nodes and expression of OX40 ligand, both of which were inhibited in TSLP receptor knockout mice. Xylene promoted the picryl chloride-induced thickening of the ear and IL-4 production, which were reversed in TSLP receptor knockout mice. ***Conclusion:*** Xylene induced TSLP production, resulting in an exacerbation of allergic inflammation. Thus, xylene might be a good tool for examining the roles of TSLP in eliciting allergy in experimental animals.

Introduction

Recently, the number of patients with allergic diseases has been increasing. Exposure to several chemical compounds in the environment might worsen allergies. However, it remains unclear which chemicals modify immune responses and how.

Thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine produced by epithelial cells [1] and mast cells [2], plays an important role in the initiation of allergic inflammation [3]. TSLP production is increased at inflamed sites in patients with severe asthma [4], atopic dermatitis [5] and allergic rhinitis [6]. The allergic inflammation in an animal model of asthma was significantly

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suppressed in TSLP receptor-deficient mice [7]. In addition, the intratrachial administration of Fc-TSLP receptor-fusion protein or anti-TSLP receptor significantly reduced eosinophil infiltration, hyperplasia, and Th2 cytokine production [7, 8]. Lung-specific expression of TSLP induced asthma-like airway inflammation [9], and skin-selective expression and intradermal injection of TSLP induced atopic dermatitis [10, 11]. Thus, an excess of TSLP is enough to cause allergic inflammation.

TSLP induces the activation of immature dendritic cells, recruitment of mature dendritic cells into lymph nodes, and expression of the OX40 ligand (OX40L) [12], which triggers the differentiation of allergen-specific naïve CD4+ T cells into inflammatory Th2 cells that produce IL-4, IL-5, IL-13 and TNF-α [12, 13].

It has been reported that epithelial cells (e.g. airway epithelial cells and keratinocytes) and mast cells produce TSLP when stimulated with antigens, cytokines and Toll-like receptor ligands [2, 14–16]. The production of TSLP by epithelial cells was significantly enhanced by TNF-α and/or IL-4 [14, 15]. Authors have reported that the application of 12-O-tetradecanoylphorbol-13-acetate (TPA) to the earlobes of mice induced the expression of TSLP mRNA [17]. The findings indicated that the production of TSLP could be induced by nonimmunological stimulants such as chemical compounds.

The first cells to interact with chemical compounds in the environment are the epithelial cells of the respiratory system, digestive tract and skin. Therefore, chemicals that promote Th2-type reactions and worsen allergic inflammation might induce TSLP production by epithelial cells. To clarify the involvement of TSLP in the induction of Th2-type reactions, we used our novel allergic inflammation model using picryl chloride (PiCl), a contact-sensitizing chemical [17]. PiCl induced Th1-dominant contact hypersensitivity in the mice treated with cyclophosphamide, which causes blood eosinophils [18] and decreases the number of regulatory T cells [19]. However, the application of TPA after the sensitization with PiCl, which induces TSLP production, shifted the PiCl-induced allergic inflammation from a delayed-type response to a biphasic response, increased the infiltration of eosinophils, and the cytokine milieu from Th1 to Th2 [17]. Thus, this model is useful for detecting the chemical such as TPA, which shifts the cytokine milieu from Th1 to Th2 by producing TSLP.

In this study, the activity of various organic solvents to elicit TSLP production was examined in vivo and xylene was found to potentially augment Th2-type allergic responses by inducing TSLP production.

Animals and Methods

Animals

Male BALB/c mice and C57BL/6 (5 weeks old), WBBF1 wild-type, and W/W^{v} mice were purchased from SLC (Shizuoka, Japan). The generation of TSLP receptor knockout mice (C57BL/6 background) has been described previously [20]. BALB/c TNF-α knockout mice (BALB/c background) were established from original TNF-α knockout mice [21]. IL-4 receptor α-chain gene knockout mice (BALB/c background) [22, 23] were purchased from Immuno-Biological Laboratories, Co. Ltd. (Takasaki, Japan). The study protocol was approved by the Institutional Animal Care and Use Committee of Tohoku University (Permission No. 20-Pharma-Animal-22).

Assay of TSLP Production Triggered by Organic Solvents

The organic solvents used were benzene, chloroform, ethyl acetate, toluene, xylene, xylene isomers, 1,3,5-trimethylbenzene, and 1,2,4-trimethylbenzene (Wako Pure Chemical Industries, Osaka, Japan) and TPA (Sigma-Aldrich, St. Louis, Mo., USA). Twenty microliters of solvent or a 0.04-μg/μl TPA solution (3:1 in acetone:ethanol) was painted on the right earlobe of mice. Earlobe tissue was then punched out (diameter 5 mm) at a specified time and weighed.

Determination of the Expression of mRNA for TSLP in Earlobes

The total RNA of tissues was extracted using a GenElute Mammalian Total RNA Kit (Sigma-Aldrich) according to the manufacturer's instructions. The extracted RNA (0.5 μg) was reverse-transcribed by using M-MLV reverse transcriptase (Invitrogen Co., Carlsbad, Calif., USA). PCR amplification of the cDNA was performed with *Taq* polymerase (Takara, Ohtsu, Japan) and specific primers. The sequences of the primers used and PCR conditions for the amplification of TSLP cDNA were: (forward) 5′-GAC AGC ATG GTT CTT CTC AG-3′ and (reverse) 5′-CTG GAG ATT GCA TGA AGG-3′, 40 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min. The murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene (a housekeeping gene) was used as an internal standard. The sequences of primers used for the amplification of GAPDH cDNA were: (forward) 5′-TGA TGA CAT CAA GAA GGT GGT GAA G-3′ and (reverse) 5′-TCC TTG GAG GCC ATG TAG GCC AT-3′. PCR was performed for 27 cycles, denaturation at 94°C for 0.5 min, annealing at 57°C for 1 min, and extension at 72°C for 2 min.

Determination of TSLP Protein in the Tissue

The tissue samples were homogenated at 4°C in 10 vol of phosphate-buffered saline by a Beads Cell Disrupter (MS-100, Tomy Digital Biology Co., Tokyo, Japan). The concentration of TSLP in the supernatant of the homogenate was determined by ELISA (R&D Systems, Minneapolis, Minn., USA). The supernatant contains unidentified substances which nonspecifically bind to the biotin-labeled enzyme of the ELISA kit. Therefore, the level of TSLP in the supernatant was measured with or without a secondary antibody to recognize nonspecific binding. The value obtained with the homogenate of untreated earlobes was then subtracted from the data to give the increase in the level of TSLP.

Histological Analysis

The earlobe tissue was excised 24 h after the painting of xylene and immediately fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were immunostained or stained with hematoxylin and eosin. The immunostaining of TSLP was performed using a rabbit anti-human TSLP antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA) and the avidin-biotin-peroxidase system (Vector Laboratories Inc., Burlingame, Calif., USA).

Determination of OX40L mRNA Levels in Cervical Lymph Nodes

Xylene (20 μl) was painted on the right ear of mice once or once a day for 7 days. Twenty-four hours after the last painting, the cervical lymph nodes were excised and weighed. cDNA was prepared as described above. The sequences of the primers used and conditions for the amplification of OX40L cDNA were as follows: (forward) 5′-CAG AGG AGC AGT TAC CAG AT-3′ and (reverse) 5′-CAG GAG CAT TTA CAG TCA GG-3′, 31 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. The levels of mRNA for OX40L and GAPDH were quantitated by scanning densitometry, and the density ratio of the OX40L mRNA to the GAPDH mRNA was calculated.

Induction of PiCl-Induced Allergic Inflammation and Determination of IL-4

The effects of xylene on PiCl-induced contact dermatitis were examined as reported [17]. Briefly, cyclophosphamide (Sigma-Aldrich) was dissolved in saline and injected subcutaneously at a dose of 150 mg/kg into TSLP receptor knockout mice and wild-type mice. Two days later, mice were sensitized with 50 μl of a 7% (w/v) PiCl solution (Nacalai Tesque, Kyoto, Japan; 3:1 in acetone:ethanol; day 0). Thereafter, 20 μl of xylene was applied to the same site twice (days 5 and 10). On day 12, mice were challenged with 20 μl of a 1% (w/v) PiCl solution by painting it on the right earlobe. Ear thickness was measured with a dial thickness gauge (Peacock, Ozaki, Tokyo, Japan) at a specified time. A homogenate of earlobe tissues was prepared as described above and the level of IL-4 was determined by ELISA (e-Biosciences Inc., San Diego, Calif., USA). Nonspecific binding was subtracted as described above.

Statistical Significance

The statistical significance of the results was analyzed with the Dunnett test or the Student-Newman-Keuls test for multiple comparisons. The results were confirmed by at least three independent sets of experiments.

Results

TSLP Production Induced by Organic Solvents

Various organic solvents were painted on the right earlobes of mice. Consistent with previous findings [17], TPA induced the expression of TSLP mRNA in the earlobes excised 4 h after the painting (fig. 1). Under these conditions, the application of toluene and xylene, but not chloroform or ethyl acetate, induced the expression of TSLP mRNA (fig. 1). The effects of aromatic compounds on the production of TSLP protein were then examined. TPA also induced the increase in TSLP protein in the homogenate of earlobe tissue excised 24 h after the painting, and the amount of TSLP (increase vs. untreated) was 515 ± 18 pg/ml. Xylene but not benzene increased the level of TSLP in the homogenate (table 1). The level of activity of toluene was in between that of xylene and benzene. Because the xylene used was a mixture of 3 isomers, *o*-xylene, *m*-xylene and *p*-xylene (1:5:3), the activity of each isomer was determined. Interestingly, *m*-xylene induced

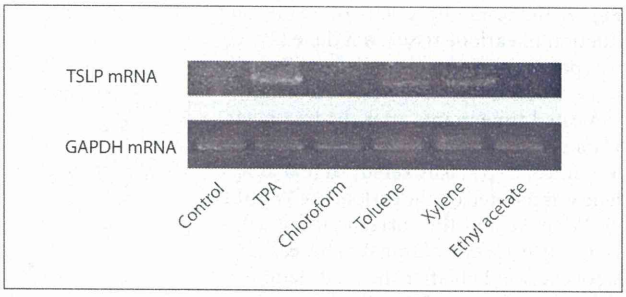

Fig. 1. Effects of chemical compounds on the expression of TSLP mRNA. Twenty microliters each of a 0.04 μg/μl TPA solution, chloroform, toluene, xylene and ethyl acetate were painted on the earlobes of BALB/c mice. Four hours later, total RNA was extracted from the excised earlobe tissue and the mRNAs for TSLP and GAPDH were detected by RT-PCR.

Table 1. Induction of TSLP by organic solvents

Organic solvent	TSLP (increase vs. untreated), pg/ml
No solvent	0 ± 74
Benzene	53 ± 96
Toluene	249 ± 68*
Xylene	579 ± 128**
o-Xylene	154 ± 68
m-Xylene	801 ± 145**
p-Xylene	345 ± 90**
1,2,4-Trimethylbenzene	2,178 ± 279**
1,3,5-Trimethylbenzene	1,531 ± 184**

Organic solvents (20 μl) were painted on the right earlobe of BALB/c mice. The earlobe tissue was excised 24 h after the painting and the level of TSLP in the tissue homogenate was determined by ELISA. Data are shown as the mean ± SEM for 6–10 mice. Statistical significance: * $p < 0.05$ and ** $p < 0.01$ vs. with no solvent.

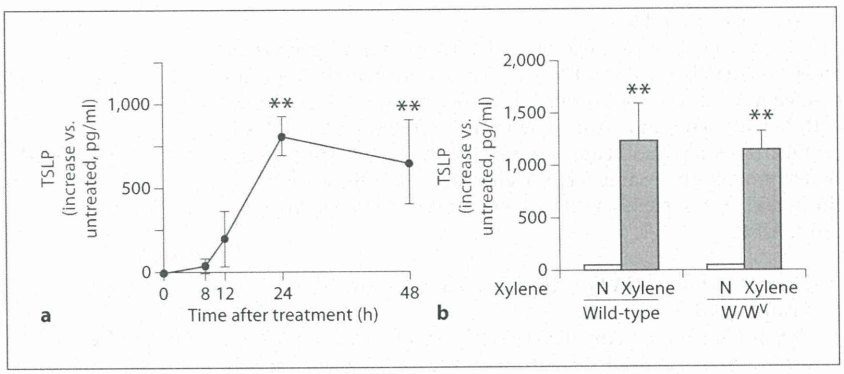

Fig. 2. Induction by xylene of TSLP production in earlobe tissue. **a** Xylene (20 μl) was painted on the right earlobe of BALB/c mice. TSLP levels were determined at the indicated time points after the treatment. Data are shown as the mean ± SEM for 4–5 mice, ** $p < 0.01$ versus time 0. **b** Xylene was painted on the earlobes of WBBF1 W/W^v mice and the corresponding wild-type mice (grey columns). The earlobes were excised 24 h after the treatment. N = Non-treated group. Data are shown as the mean ± SEM for 5 mice, ** $p < 0.01$ versus the corresponding N.

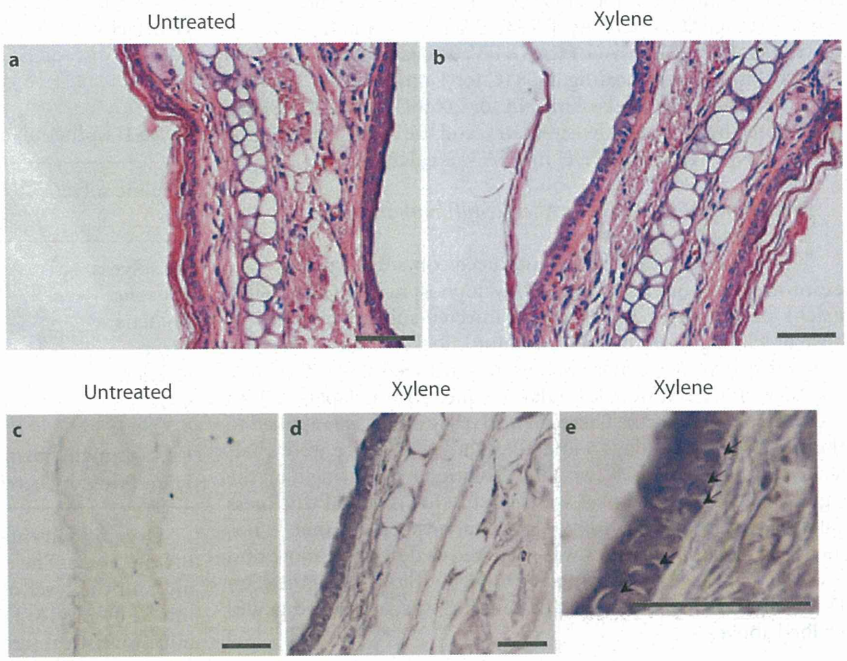

Fig. 3. Histochemical analysis of TSLP-producing cells. Xylene was painted on the right earlobe of BALB/c mice. The tissue of right earlobe tissue (**b**, **d**, **e**) and the left earlobe (untreated ear, **a**, **c**) were excised 24 h after the treatment. Tissue sections were stained with hematoxylin and eosin (**a**, **b**) and anti-TSLP antibody (**c**–**e**). **e** This is a higher magnification of **d**. The positive cells were indicated with arrows. The scale bar represents 100 (**a**, **b**) and 50 μm (**c**–**e**).

the production of TSLP much more extensively than did *o*-xylene (table 1). Furthermore, both 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene also triggered marked TSLP production (table 1). Since the xylene identified in indoor environments is a mixture of 3 isomers, we used xylene as a chemical inducer of TSLP production in subsequent experiments.

TSLP-Producing Cells

The level of TSLP in the xylene-treated earlobes slowly increased and reached a maximum at 24 h (fig. 2a). The painting of xylene on the earlobes of mast cell-deficient W/W^v mice caused the production of TSLP at 24 h to almost the same extent as in the corresponding control mice (fig. 2b). The painting of xylene almost did not induce the infiltration of leukocytes (fig. 3a, b). The immunostaining with anti-TSLP disclosed that the TSLP-producing cells were mainly located in the epithelial layer of the xylene-treated ear (fig. 3d, e).

Involvement of TNF-α and IL-4 in the Xylene-Induced Production of TSLP

To clarify whether TNF-α and IL-4 enhanced xylene-induced production of TSLP, xylene was painted on the

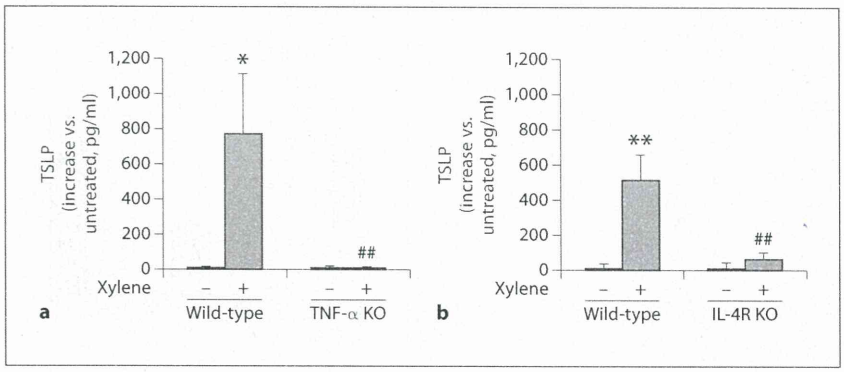

Fig. 4. Xylene-induced production of TSLP in earlobes of TNF-α knockout mice and IL-4 receptor knockout mice. Xylene (20 μl) was painted on the right earlobe of BALB/c wild-type mice and TNF-α knockout mice (TNF-α KO; **a**) and IL-4 receptor knockout mice (IL-4R KO; **b**). The earlobes were excised 24 h later. The concentration of TSLP in the supernatant of the earlobe homogenate was determined by ELISA. Data are shown as the mean ± SEM for 7 mice, * $p < 0.05$, ** $p < 0.01$ versus without xylene (–), ## $p < 0.01$ versus with xylene (+) in wild-type mice.

earlobes of TNF-α knockout mice and IL-4 receptor knockout mice. In these knockout mice, xylene-induced TSLP production was significantly diminished (fig. 4).

Increase in the Weight of Cervical Lymph Nodes and Expression of OX40L on the Painting of Xylene

To clarify whether or not xylene caused the production of a significant amount of TSLP to activate dendritic cells, the weight of the cervical lymph node and the expression of OX40L were determined. Although not significantly increased 1 day after the painting of xylene, the weight of the cervical lymph node was significantly greater following repeated treatment with xylene for 7 days (fig. 5a). The xylene-induced increase was smaller in C57BL/6 mice (fig. 5b) than in BALB/c mice, and partially but significantly reduced in TSLP receptor knockout mice (fig. 5b). In addition, the level of OX40L mRNA in the lymph node was apparently increased by the repeated treatment with xylene in wild-type mice but not in TSLP receptor knockout mice (fig. 5c, d).

Exacerbation of PiCl-Induced Allergic Inflammation by Xylene

Finally, the possibility was examined that xylene exacerbated antigen-induced allergic inflammation via TSLP production. As shown in figure 6a, the painting of xylene on the same earlobes 5 and 10 days after the sensitization with PiCl enhanced the PiCl-induced increase in ear thickness. The levels of IL-4 in the earlobe tissue 24 h after the challenge were also increased by the application of xylene (fig. 6b). The enhancement by xylene of ear swelling and IL-4 production was not observed in TSLP receptor knockout mice (fig. 6).

Discussion

TSLP is a master cytokine inducing Th2-type allergic inflammation [3]. In our study, some organic chemicals, i.e. volatile solvents used in paints and glues, induced production of TSLP, resulting in an exacerbation of allergic inflammation.

Among the organic solvents tested, xylene, especially *m*-xylene, 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene significantly caused the production of TSLP in vivo (fig. 1, table 1). Interestingly, the activity to induce TSLP production was highly dependent on the position of methyl groups on the benzene ring. Namely, *m*-xylene induced much more extensively the production of TSLP than did *o*-xylene. These findings suggested that xylene triggered TSLP production by binding to a specific protein in a structure-dependent manner, and not via physical and/or chemical toxicity.

The TSLP-producing cells were mainly epithelial cells and mast cells [1, 2]. In addition, mast cells induced epithelial TSLP production in a model of allergic rhinitis [24]. However, the painting of xylene on the earlobes of mast cell-deficient W/W^v mice caused the production of TSLP to almost the same extent as in the corresponding control mice (fig. 2b), indicating that mast cells were not involved in the xylene-induced production of TSLP. The painting of xylene almost did not induce the infiltration of leukocytes, indicating that the cells existing in the skin might be producing TSLP. The immunostaining analysis disclosed that the TSLP-producing cells were mainly located in the epithelial layer of the xylene-treated ear (fig. 3d, e). These findings suggested that the cells, such as keratinocytes, in the epithelial layer produced TSLP in response to xylene.

TNF-α and IL-4 only slightly induced production of TSLP but enhanced Toll-like receptor ligand-induced

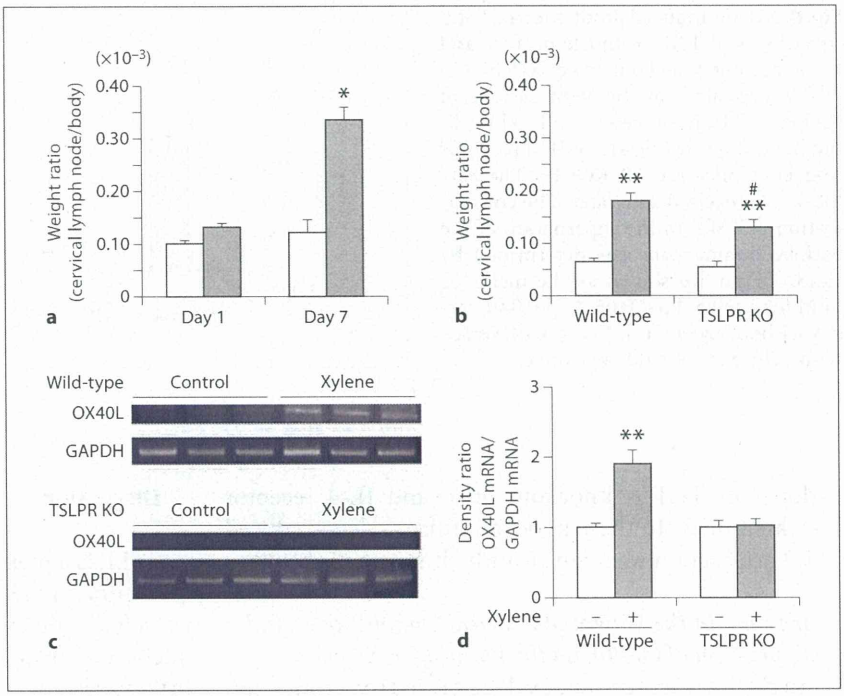

Fig. 5. Xylene-induced increase in the weight of the cervical lymph node and expression of OX40L. **a**, **b** Xylene was painted on the earlobe of BALB/c mice once or once a day for 7 days (**a**) or of TSLP receptor knockout mice and the corresponding wild-type mice (C57BL/6) once a day for 7 days (**b**). The cervical lymph nodes were excised 24 h after the last treatment. The weight of the cervical lymph node relative to body weight was determined. Grey columns indicate the xylene-treated group and open columns indicate the untreated group. Data are shown as the mean ± SEM for 5 mice, * $p < 0.05$, ** $p < 0.01$ versus the corresponding untreated group, # $p < 0.05$ versus the corresponding wild-type mice. **c**, **d** The expression of OX40L mRNA in the lymph nodes excised from wild-type mice and TSLP receptor knockout mice treated with xylene for 7 days was determined by RT-PCR. Data indicate the results for 3 mice in each group. The density ratio of OX40L mRNA to GAPDH mRNA was calculated (**d**). The mean density ratio of the untreated group was set to 1.0. Data are shown as the mean ± SEM for 3 mice, ** $p < 0.01$ versus the corresponding untreated group.

production in vitro [14–16]. No increase in the levels of these cytokines in the earlobe tissue obtained 4–24 h after the painting of xylene was detected (data not shown). However, xylene-induced production of TSLP was significantly diminished in TNF-α knockout mice and IL-4 receptor knockout mice (fig. 4), indicating that xylene elicited TSLP production via costimulation with basal or low levels of TNF-α and IL-4. The producing cells of TNF-α and IL-4 might be the responding cells to xylene, but they were not identified via the immunostaining (data not shown). These findings also suggested that there is a common pathway triggered by xylene and Toll-like receptor ligands. The molecular mechanisms by which xylene induces TSLP production are under investigation.

TSLP induces the expression of OX40L on dendritic cells [12] and the proliferation of Th2 lymphocytes [12, 13]. In the previous model, xylene increased the weight of the cervical lymph node and the expression of OX40L (fig. 5). Because these responses were reduced in TSLP receptor knockout mice, it was likely that xylene triggered the activation of dendritic cells via the production of TSLP, resulting in an increase in the proliferation of lymphocytes in the cervical lymph nodes. Because the weight of the lymph node was increased by xylene in TSLP receptor knockout mice, the possibility that xylene induced the production of cytokines other than TSLP, which caused lymphocytes to proliferate, could not be ruled out.

Some chemical compounds, such as formalin, bind to proteins and act as a hapten to induce allergies. The antigenicity of xylene itself, however, has not been reported. It is possible that xylene exacerbated the antigen-induced

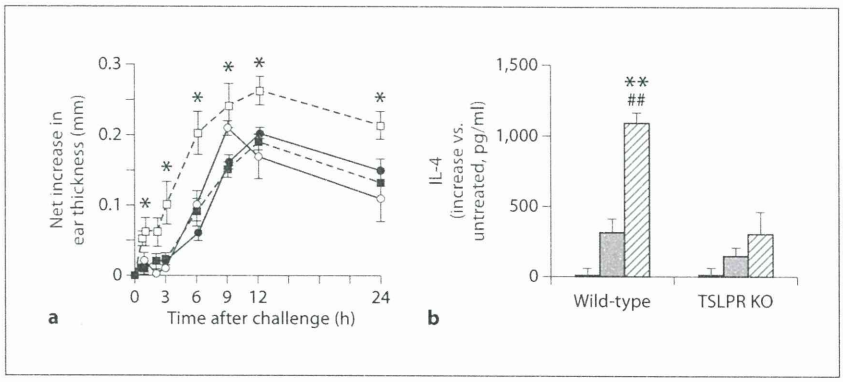

Fig. 6. Exacerbation of PiCl-induced allergic inflammation by xylene. **a** C57BL/6 wild-type mice (squares) and TSLP receptor knockout mice (circles) were pretreated with cyclophosphamide (150 mg/kg) on day –2 and 50 μl of a 7% (w/v) PiCl solution was painted on the right earlobe on day 0. The sensitized mice were treated with xylene at the same site on days 5 and 10 (open symbols) or left untreated (closed symbols). On day 12, mice were challenged with 20 μl of a 1% (w/v) PiCl solution painted on the right earlobe. Earlobe thickness was measured 0–24 h after the challenge. Ear thickness before the challenge was subtracted from the data. Data are shown as the mean ± SEM for 5 mice, * $p < 0.05$ versus the corresponding PiCl-treated group. **b** The concentration of IL-4 in homogenate of the earlobe tissue excised 24 h after the PiCl challenge was determined by ELISA. Open columns, hatched columns and grey columns indicate the untreated group, PiCl-treated group and PiCl + xylene-treated group, respectively. Data are shown as the mean ± SEM for 5 mice, ** $p < 0.01$ versus the corresponding untreated group, ## $p < 0.01$ versus the corresponding PiCl-treated group.

allergic inflammation via TSLP production. Authors have reported that the application of TPA aggravated PiCl-induced allergic inflammation [17]. In that model, TPA elicited the production of TSLP and shifted the cytokine milieu from Th1 to Th2 [17]. In our study, xylene, which was used instead of TPA, enhanced the PiCl-induced thickening of the ear and IL-4 production (fig. 6). Xylene failed to induce a biphasic response which was observed by PiCl plus TPA [17]. The discrepancy might be caused by the use of a different mouse strain. In the previous study, we used Balb/c mice, i.e. Th2-oriented mice, but in this study, we used C57BL/6 mice, i.e. Th1-oriented mice, because the TSLP receptor knockout mice we used had a C57BL/6 background. The finding that the xylene-induced enhancement of these responses was reversed in TSLP receptor knockout mice suggested that xylene exacerbated the PiCl-induced allergic inflammation via production of TSLP, which elicited an enhancement of Th2-type immune responses as determined from IL-4 levels.

Xylene, 1,3,5-trimethylbenzene and 1,2,4-trimethylbenzene are volatile solvents of paints and glues and often detected in indoor environments [25]. Recently, it was reported that repeated exposure to a cigarette smoke extract induced TSLP production and promoted antigen-induced Th2-type immune responses and airway inflammation [26]. Therefore, the inhalation of these organic solvents may also induce TSLP production in respiratory tissue and exacerbate asthma and allergic rhinitis. Authors have now begun studying this possibility.

In conclusion, xylene induced the production of TSLP and aggravated allergic inflammation. This is the first report of a pure chemical compound detected in the environment triggering TSLP production and worsening allergic inflammation in vivo. The models employed here would be useful for detecting chemicals that exacerbate allergic inflammation without antigenicity. In addition, xylene might be a good tool for examining the role of TSLP in eliciting allergy in experimental animals.

Acknowledgements

This study was supported in part through a grant of the Long Range Research Initiative (LRI) by the Japan Chemical Industry Association (JCIA), Grant-in-Aid for Challenging Exploratory Research (22659025) from the Japan Society for the Promotion of Science, and by the Division of Intramural Research, National Heart, Lung, and Blood Institute, NIH.