(2) 喫煙の口腔影響の禁煙導入への用い方

禁煙の勧め方の基本は「禁煙はあなたの健康のために行う優先順位の最も高い方法である」ことを説明することが前提である。禁煙を勧めることは医療従事者の責務だとは思うが、最初はその勧め方がわからないため心配するのが当たり前である。禁煙のステージ理論では、喫煙から長期の禁煙に至る経過を前熟考期・熟考期・準備期などのステージを経るプロセスと捉えて、ステージに適合した禁煙の勧めを行う。一方、米国で2000年に出版された禁煙治療ガイドライン[11]では、禁煙治療の一般的なスケジュールとして5つの手順（頭文字をとって5A）を示している。5Aの手順は口腔衛生指導の手順と似ているので、歯科診療に導入しやすい。3番目には喫煙者を禁煙する意思のある者とない者にわける評価を行う（Assess）。

禁煙の意思がない者に用いる手法として5つの方法（5R）を示している（表3）。歯科では前述したように喫煙者本人の口腔の状況や歯科治療の効果と喫煙を関連づけ（Relevance）、将来の危険性（Risks）とともに繰り返し（Repetition）説明することが日常の臨床で行うことができる。特に喫煙に関係する個別の内容（表4）を示すことは、どの喫煙する患者にも適用できる歯科専門職としての利用価値の高いものであるので、是非とも試していただきたい。

日常診療では患者に対して、こうした多種多様な喫煙の口腔への影響を、短時間で、簡便に、しかも効果的に示すことができる教材があれば便利である。そこで、われわれは、タバコ箱の警告表示で実証された喫煙者の禁煙の意思に及ぼす画像による警告の効果をヒントにして、喫煙の口腔影響を口腔写真や影響の大きさをグラフで表し、歯科医師や歯科衛生士が説明する文言も含めたカラーチャートを作成して配布している（図5）。

(3) 禁煙支援と禁煙治療

喫煙者の多くは禁煙したいが、なかなかやめられない。その理由は、喫煙には2種類の依存、心理行動依存とニコチン薬理依存があるからである。前者にはカウンセリング療法、後者には薬物療法を用いることが有効な対応策である。禁煙により長期的には口腔の健康のリスクが軽減するだけでなく歯科治療効果が向上する。一般に、禁煙の効果が現れてくるのには十年以上の歳月を必要することがわ

表3　米国禁煙ガイドラインにおける患者の禁煙意思に対応した基本的な禁煙のアプローチ

禁煙治療の手順と口腔衛生指導			禁煙意思の低い患者への対応（5Rの内容）	
5A手順	5Aの内容	口腔衛生指導		
Ask	喫煙状況	ブラッシング・甘味摂取	Relevance	関連づける
Assess	禁煙意思の評価	予防意識・プラーク評価	Risks	リスクを話す
Advise	禁煙の助言	ブラッシング・代用甘味料	Rewards	禁煙でよい事がある
Assist	禁煙の支援	歯磨き技術・キシリトール	Roadblocks	禁煙の障壁
Arrange	フォロー	フォローアップ	Repetition	反復助言

表4　歯科診療における禁煙導入の機会と内容

歯科診療の機会		禁煙の導入に用いる内容
問診時の会話		喫煙習慣、禁煙経験、口臭、歯の早期喪失・歯周病・口腔がんのリスク
口腔診査の結果説明	歯	歯の喪失のリスク、歯の着色
	歯周組織	歯根膜細胞へのニコチンの影響、セメント質ニコチン沈着、歯槽骨の吸収、アタッチメントロス、歯肉微小循環、歯肉の出血に気がつかない、歯肉の着色
	口腔粘膜	口腔がん、白板症、その他喫煙が関連する口腔粘膜の異常
充填時・補綴物装着時		歯の早期喪失に伴う充填物、補綴物の維持時間短縮
歯周基本治療、外科処置実施前		非外科的歯周治療および外科的歯周治療の予後不良、前歯部充填物の着色
インプラントの診査・説明		インプラント失敗の可能性
抜歯実施後の説明		抜歯後の創傷治癒の遅延
リコール時		歯の早期喪失、歯周病の進行、歯や歯肉の着色、口臭
スケーリング施術時		患者は上記の全ての内容について、聞きやすい姿勢になっている。

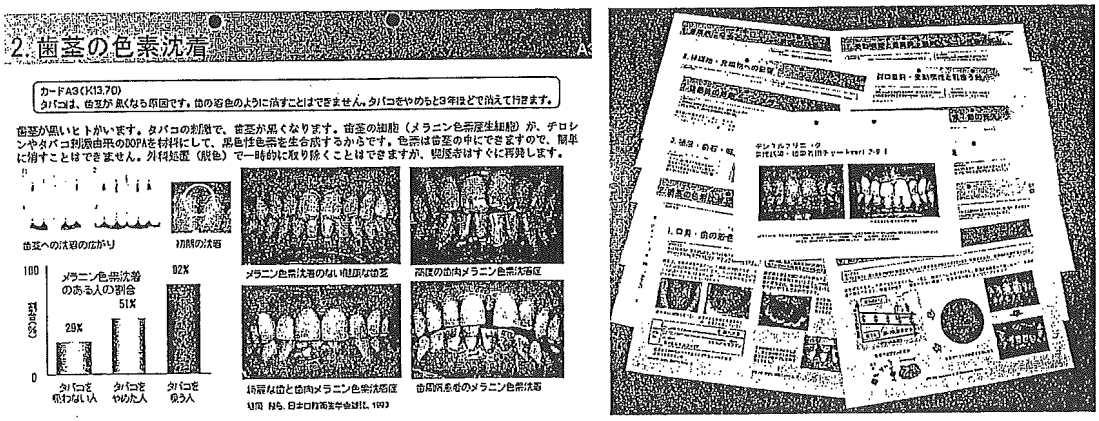

図5　日常診療で簡便に繰り返し、効果的に患者を禁煙に誘導するためのカラーチャート
(科学的根拠の説明や患者に説明する標準的な文言も含まれている)

かっている。しかし、最近発表された歯周治療への影響では、歯周治療を開始する前に禁煙した場合には1年後に禁煙を継続していた者より治療効果が向上したことが報告された[12]。

現在、禁煙支援や禁煙治療にかかわる費用は健康保険の対象にはなっていない。現状ではニコチン代替え薬はOTC(over-the-counter)薬あるいは自費扱いの処方箋薬(処方箋料は自費であるが混合診療とはならない)で禁煙

指導料も自費であるため、医療における禁煙治療が普及しない理由だとされる。禁煙は、喫煙による疾病に関わる莫大な医療費を節約することがわかっているが、わが国の医療保険制度は病気の治療に対して報酬が支払われる制度であるので医療保険の適用にはならない。しかし、喫煙を継続することが歯科治療の効果を低下させ、禁煙が治療効果を回復させるのであれば、歯科医師による禁煙指導は歯科医療保険の制度でも補填されるべきである。

5. おわりに

喫煙は口で行われる。有害物質を多種多量に含むタバコの煙は口腔を通過し、あるいは、口腔に留まる。口腔を通過した有害物質は体循環により再び口腔にもどり口腔の健康や歯科治療に悪影響を及ぼす。喫煙が口と関連することは明白なのに、歯科医師が喫煙に注目する機会は少なかった。

禁煙は健康の専門家の役割のひとつである。そして、歯科医療に関わる者は喫煙者に禁煙を勧めることが務めである、との認識が醸成されつつある段階である。今、ひとり一人の歯科医師が主治医となる患者の喫煙に対して、自らの姿勢とこれからの行動について少しの時間考えていただければ、次世代の歯科医師のさらなる活躍につながる第一歩になると思う。

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JSPP会員各位へ

JSPP研究助成事業委員会からのお知らせ

第6回平成17年度JSPP研究助成対象研究が決定しました。

・研究課題：小児歯科臨床におけるQLF（光誘発蛍光定量法）の有用性の検討について
・研究者名：南　真紀先生（明海大学小児歯科学講座）

研究結果については平成18年第18回JSPP全国集会で発表の予定です。
なお今回から助成対象を従来の「小児歯科の臨床に直結した課題」を多少広義に捉え範囲が拡げられました。多様な研究対象のご応募が期待されます。
平成18年度の募集を始めます。下記の事項をご記入の上、応募ください

1) 研究課題名：
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3) 研究課題提案者氏名：
4) 研究課題提案者連絡先：（住所・電話）
5) 提案者が推薦する研究者氏名
（推薦できる研究者あるいは団体がもしありましたら、お書きください）

■応募先　JSPP研究助成事業委員会
連絡先：〒592-0002 大阪府高石市羽衣1丁目6－31　大橋小児歯科医院　大橋健治
TEL & Fax　072-265-3231　ohadcc@wondernet.ne.jp

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Association of lip pigmentation with smoking and gingival melanin pigmentation

Satoru Haresaku, Akihito Tsutsui, Takeshi Watanabe and Takashi Hanioka
Department of Preventive and Public Health Dentistry, Fukuoka Dental College, Fukuoka, Japan

Key words: Lip, Gingiva, Pigmentation, Melanin, Smoking,

Abstract

Objective: We investigated association of lip pigmentation with smoking and melanin pigmentation in the gingiva.

Design: Case-control study

Setting: Health check-up in an institute

Subjects and Methods: Photos of 223 males worked in an institution were assessed in terms of pigmentation in lip and gingiva.

Main outcome measures: Prevalence and scores of lip and gingival pigmentation, Smoking status

Results: Among subjects displaying lip and gingival pigmentation, 73% and 87%, respectively, were current smokers, whereas 33% and 27% of individuals lacking pigmentation were current smokers, respectively. Odds ratios of current smoking relative to lip and gingival pigmentation were 5.6 (95% confidence interval: 2.8-11.1) and 17.0 (8.1-36.0), respectively. Daily consumption, duration of smoking and lifetime exposure exhibited significant correlation with scores of lip and gingival pigmentation ($P<0.0001$). Odds ratios increased in lip and gingival pigmentation upon exposure. In current smokers, scores of lip and gingival pigmentation demonstrated meaningful correlation ($P<0.0001$); moreover, 95% of participants with lip pigmentation were positive for gingival pigmentation.

Conclusion: These results indicated the presence of a striking association between smoking and pigmentation in the lip and gingiva, which was stronger with respect to gingival pigmentation. Health professionals could educate smokers utilizing visible symptoms in the lip and gingiva.

Introduction

Brownish or black discoloration, i.e., melanin pigmentation, which occurs as a solitary unit or as a continuous ribbon in gingiva, is distinguishable from other forms of oral pigmentation (Cicek and Ertas, 2003). The prevalence of melanin pigmentation in the gingiva differs by ethnic group, which is indicative of a hereditary connection (Steigmann S, 1965; Fry and Flmeyda, 1968; Hedin, 1977; Axell and Hedin, 1982; Araki et al., 1983; Hedin and Larsson, 1984; Hanioka et al, 1993; Ünsal et al., 2001; Sarswathi et al., 2003). Gingival pigmentation is evident in subjects receiving antimalarial drugs (Dencker et al., 1976; Main, 1988); however, this phenomenon is rare. Melanin pigmentation derives from melanin granules in gingival tissue, which are produced in melanosomes of melanocytes (Hedin and Larsson, 1984). Melanin is synthesized from tyrosine and dihydroxyphenylalanine (DOPA) via dopaquinone by oxidation of tyrosinase (Halaban et al., 2001).

Gingival pigmentation has been examined in terms of association with smoking in various countries, including Israel (Steigmann S, 1965), Great Britain and Sweden (Axell and Hedin, 1982), Japan (Araki et al., 1983; Hanioka et al, 1993), Thailand and Malaysia (Hedin and Axell, 1991), Turkey (Ünsal et al., 2001) and India (Sarswathi, 2003). Excessive melanin pigmentation is correlated with smoking; thus, smoking may stimulate melanin production in gingival tissue. The stimulatory effect could occur as a result of the high affinity function of nicotine (Claffey et al., 2001) and benzpyrene (Roberto et al., 1996) in tobacco smoke relative to melanin. Additionally, a dose-response relationship was detected (Araki et al., 1983). Disappearance of gingival pigmentation was observed following reduction in smoking (Hedin et al., 1993). These findings suggest a causal association between smoking and gingival pigmentation.

Gingival pigmentation is visible because of the presence in the labial area of anterior teeth (Hedin, 1977; Axell and Hedin, 1982; Hanioka et al., 1993: Sarswathi et al., 2003). Due to specific localization of gingival pigmentation, smokers may be aware of the health consequences of smoking relative to their own bodies following proper education by health professionals. In a manner similar to gingiva, lip, which is also readily visible, may produce melanin. To the best of our knowledge, no data regarding the association between smoking and lip pigmentation appear in the literature. The objective of this study was to investigate association of lip pigmentation with smoking and gingival melanin pigmentation.

Subjects and methods

Digital photos of lip and the labial aspects of frontal teeth, which were produced in a standardized manner (D70, Nikon, Tokyo), were obtained from 223 males (31.8 ± 8.9 years of age average ± S.D.)

employed by an institute at Fukuoka, Japan on the occasion of the annual health check-up. These subjects were medically healthy individuals. Digital images were stored on electronic media, followed by subsequent reproduced on a computer display. These reproductions exhibited size similar to that of the actual mouth. The number of females in the workplace and the smoking rate among females in Japan are small in comparison to those of males; consequently, females were excluded from this study.

Lip pigmentation was scored dichotomously (0, 1) for existence of diffuse form of black or brownish discoloration in the vermilion boarder. Pigmentation was scored in individual sextant of the lip: subsequently, total score was calculated. This study first addressed lip pigmentation in relation to smoking in a population of certain size; as a result, we examined reliability of the classification of lip pigmentation. Assessment of pigmentation was calibrated by two examiners employing representative photos. The examiners then evaluated 240 sections of lip in 40 photos (6 sextants per individual). K statistic for existence of lip pigmentation was 0.88, which indicated that inter-examiner agreement was excellent and that the subjective evaluation of lip pigmentation was reliable.

Gingival pigmentation was scored in each jaw according to classification of the Melanin Index (Hedin, 1977, Figure). The index classified pigmentation as follows: 0, no pigmentation; 1, one or two solitary unit(s) of pigmentation in papillary gingiva without formation of a continuous ribbon between solitary units, 2; more than three units of pigmentation in papillary gingiva without formation of a continuous ribbon; 3, one or more short continuous ribbons of pigmentation, and 4, one continuous ribbon including the entire area between canines. Total scores of upper and lower jaw were used for analysis.

Observations of lip and gingival pigmentation were performed separately. Smoking status was withheld from the examiner of pigmentation. Smoking status was defined with a questionnaire: CS denotes an individual who currently smokes more than 100 pieces; FS describes an individual who previously smoked more than 100 pieces but does not smoke currently; NS refers to an individual who has never smoked or who had smoked no more than 100 pieces.

Melanin pigmentation is a visible symptom; thus, smokers could readily recognize adverse effect of smoking. If CS could be identified on the basis of lip or gingival pigmentation, smokers may actually experience the negative effect of smoking prior to onset of a serious illness attributable to smoking. Therefore, the potential of pigmentation as a screening measure of smoking status was examined. Generally, screening tests are utilized for early detection of non-apparent disease, whereas dichotomous classifications such as "negative" and "positive" functions, serve to distinguish corresponding disease status. In the present study, two categories, NS and CS were employed for evaluation of smoking status with respect to sensitivity and specificity (Beck, 1995). Disappearance of pigmentation was observed following reduction of smoking (Hedin et al., 1993); additionally, other variables such as duration of cessation may influence results of the evaluation. Consequently, FS was excluded from evaluation.

The protocol was approved by the *ad hoc* ethics committee of epidemiological research in Fukuoka Dental College. Informed consent was obtained from all subjects prior to the study. Associations in distribution between existence of pigmentation and smoking status and between levels of lip and gingival pigmentation were evaluated with the χ^2 test. Relationships between pigmentation scores and levels of exposure to smoking were assessed using the Spearman rank correlation. Difference in mean pigmentation scores between each category of smoking exposure and the reference (NS) was examined with the Dunnett test for multiple comparisons with contrast variable. Statistical significance was set at $p<0.05$.

Results

Among 223 subjects, 73 (33%), 112 (50%) and 28 (13%) were NS, CS and FS, respectively (**Table 1**). Lip and gingival pigmentation were apparent in 150 (67%) and 118 (53%) participants, respectively. Prevalence of pigmentation was compared according to smoking status. FS were excluded in the comparison, since disappearance of pigmentation was observed following reduction of smoking (17). Seventy-three percent of subjects exhibiting lip pigmentation were CS; in contrast, 33% of subjects lacking lip pigmentation were CS. In the case of gingival pigmentation, 87% and 27% were CS among individuals with and without pigmentation, respectively. To examine potential of melanin pigmentation as screening test for CS, sensitivity and specificity were calculated. Sensitivity and specificity of the pigmentation test for CS were 0.83 and 0.53 based on the evaluation of lip, and 0.80 and 0.81 based on that of gingiva, respectively.

Scores, prevalences and odds ratios adjusted by age (ORs) of lip and gingival pigmentation were summarized by levels of exposure to smoking including smoking status (**Table 2**). Mean scores of lip pigmentation in CS was markedly higher than that in NS; however, mean score of lip pigmentation in FS was similar to that in NS. Mean scores of gingival pigmentation were significantly higher in FS and CS than in NS. ORs of CS in lip and gingival pigmentation were 5.6 (95% confident interval: 2.8-11.1) and 17.0 (8.1-36.0), respectively, which differed significantly from those of NS. The

difference in prevalence of lip pigmentation between FS and NS was not meaningful, OR=1.4 (0.6-3.5). OR of FS in terms of gingival pigmentation was 4.5 (1.7-12.0), which was significantly different from that of NS.

Lip and gingival pigmentation were compared with respect to levels of exposure in CS involving three types of indices: daily consumption, duration of smoking and lifetime exposure. Correlation coefficients between scores of pigmentation and exposure to smoking were 0.380, 0.377 and 0.387 in lip, and 0.594, 0.640 and 0.632 in gingiva, respectively ($P<0.0001$). NS served as a reference. Mean scores of lip pigmentation for each category of exposure were also higher than those in NS, although differences were not meaningful in the minimum categories of duration of smoking and lifetime exposure. Mean score of gingival pigmentation for each level of daily consumption was greater than approximately nine times that of the corresponding score in NS. This trend was similar, 7-11 times that of NS, in other categories of exposure. ORs in lip and gingival pigmentation were significantly higher than the reference values in all categories of each index of exposure. ORs in lip and gingival pigmentation increased in accordance with level of exposure to smoking in all indices.

Levels between lip and gingival pigmentation were compared in CS and NS (**Table 3**). In CS, the correlation in levels between lip and gingival pigmentation was significant ($P<0.0001$). Gingival pigmentation was absent in 89% of those subjects lacking lip pigmentation. Ninety-five percent of subjects displaying lip pigmentation demonstrated gingival pigmentation. In NS, no meaningful association was detected in terms of levels between lip and gingival pigmentation ($P=0.1728$). Ninety-seven percent of subjects lacking lip pigmentation exhibited no pigmentation in gingiva. However, gingival pigmentation was evident in 38% of those participants characterized by lip pigmentation.

Discussion

Although meaningful correlations between smoking and gingival pigmentation have been demonstrated, the levels of association were not comparable with common measures in different populations. The results of the present study confirmed this relationship and revealed the level of association employing odds ratios: 5.6 for lip pigmentation and 17.0 for gingival pigmentation. An OR exceeding three is indicative of a relationship that is readily recognized in routine practice; consequently, smoking may be strongly connected to lip and gingival pigmentation. The powerful effects of tobacco smoke may be supported by findings pertaining to the oral effects of passive smoking. To date, periodontal disease (Aligne et al., 2003), pediatric caries (Arbes et al., 2001) and melanin pigmentation in the gingiva of children (Hanioka et al., 2005) have been described.

A dose-response relationship was also identified between levels of exposure to smoking and lip and gingival pigmentation. Furthermore, in the minimum categories of exposure to smoking, both scores and prevalence of gingival pigmentation increased relative to the level of NS and approached maximum levels. The dose-response relationship again may indicate high sensitivity of melanocytes in gingival tissue to tobacco smoking. Findings corresponding to the stimulatory mechanism of tobacco smoking in gingiva are limited (Claffey et al., 2001; Roberto et al., 1996). The highly sensitive nature of gingival melanocytes may be beneficial since young smokers could recognize a rather immediate untoward effect of smoking behavior shortly after initiation of smoking.

Strong correlation was detected between smoking and gingival pigmentation; however, lip pigmentation displayed weaker association. Association in terms of prevalence (OR) in lip pigmentation was not meaningful in FS. Furthermore, mean scores of lip pigmentation did not differ significantly between subjects derived from minimum categories of exposure and NS. NS exhibited higher prevalence of lip pigmentation (47%) in comparison to gingival pigmentation (19%); as a result, the weaker association of lip may be explained by differences in the characteristics of pigmentation. Lip may be more susceptible to sources of stimulation other than smoking. This study was the first to demonstrate a relationship between smoking and lip pigmentation.

Correlation in terms of levels between lip and gingival pigmentation was apparent in CS. Approximately 95% of smokers with lip pigmentation exhibited gingival pigmentation. Lip is readily observable in comparison to other body parts. Gingiva may also be readily accessible. Visible symptoms due to smoking in different parts of the body could potentially afford smokers an indicator via which to recognize health consequences of smoking. Furthermore, oral health professionals could elevate the awareness of smokers in dental practice. High sensitivity of gingival and lip pigmentation during screening of current smoking underscores the suitability of this method. However, clinicians should be reminded that lip and gingival pigmentation is not a flawless indicator of current smoking. To the contrary, visible symptoms of lip and gingiva may lead to unnecessary anxiety among NS and FS.

A telephone survey in Canada, where graphic warning labels on cigarette packages were first introduced, demonstrated that labels depicting lung cancer and oral diseases were extremely effective with respect to discouraging smoking (Hammond et al., 2003). The image of a mouth was selected by more smokers, especially females and young adults,

than were counterpart measures (Environic Research Group Ltd., 2001). Therefore, visible oral symptoms of smokers likely afford potential with respect to prevention and cessation of smoking.

The present study was the first to demonstrate association of lip pigmentation with smoking and melanin pigmentation in the gingiva; thus, additional investigations involving a pathological approach and employing various variables as possible confounders of smoking are required. The striking relationship between exposure of smoking and the visible symptom of pigmentation in oral and perioral conditions could potentially influence not only smoking but also oral health behaviors due to increasing awareness of oral health.

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Table 1. Distribution of subjects with or without melanin pigmentation in lip and gingiva by smoking status

		Smoking status				
	Pigmentation	Never	Current	Subtotal	Former	Total
Lip	No pigmentation	39(67)	19(33)	58(100)	12	73
	Pigmentation	34(27)	93(73)	127(100)	16	150
Gingiva	No pigmentation	59(73)	22(27)	81(100)	14	95
	Pigmentation	14(13)	90(87)	104(100)	14	118
	Total	73(39)	112(61)	185(100)	28	223

Distributions of lip and gingival pigmentation were significantly associated with smoking status (P<0.0001). Former smokers were excluded.

Table 2. Comparisons in score, prevalence and odds ratio (OR) and 95% confidence interval (CI) of lip and gingival pigmentation by levels of exposure to smoking.

	Lip pigmentation			Gingival pigmentation		
Levels of exposure (N)	Score	Prevalence (%)	OR (95% CI)	Score	Prevalence (%)	OR (95% CI)
Smoking status						
Never (73)	1.1±1.3	47	1.0(reference)	0.5±1.2	19	1.0 (reference)
Former (28)	1.0±0.9*	57	1.4(0.6-3.5)	1.8±2.2*	50	4.5 (1.7-12.0)
Current (112)	2.1±1.3*	83	5.6(2.8-11.1)	4.6±3.0*	80	17.0 (8.1-36.0)
Daily consumption (pieces)						
1-19 (37)	1.8±1.4*	76	3.9(1.6-9.7)	4.5±3.3*	76	13.5 (5.2-35.3)
20 (58)	2.0±1.3*	85	6.0(2.5-14.0)	4.6±2.9*	83	20.4 (8.3-50.6)
>20 (17)	2.8±1.3*	94	16.4(1.3-132)	4.8±3.0*	82	20.5 (4.9-85.0)
Correlation	r=0.380 p<0.0001			r=0.594 p<0.0001		
Duration of smoking (years)						
1-9 (40)	1.6±1.4	70	3.6(1.4-9.1)	3.4±2.9*	70	9.5 (3.4-26.7)
10-19 (36)	2.2±1.3*	89	8.9(2.9-27.9)	5.3±2.9*	86	27.2 (8.9-84.6)
>19 (36)	2.4±1.2*	92	9.0(2.2-37.4)	5.2±2.9*	86	37.0 (8.5-160)
Correlation	r=0.377 p<0.0001			r=0.640 p<0.0001		
Lifetime exposure (piece-years)						
1-199 (46)	1.7±1.4	72	3.8(1.6-9.2)	3.7±3.1*	72	10.9 (4.1-28.7)
200-399 (34)	2.1±1.2*	88	8.0(2.5-25.2)	5.6±2.8*	88	33.3 (9.8-113)
>399 (32)	2.5±1.2*	94	13.3(2.6-66.8)	4.8±2.8*	84	33.5 (7.8-143)
Correlation	r=0.387 p<0.0001			r=0.632 p<0.0001		

N; Number of subjects, *Significantly higher than that of never smokers

Table 3. Contingency table by score of pigmentation between lip and gingiva for current and never smokers.

Lip	Gingiva								
	Current smokers (p<0.0001)					Never smokers (p=0.1728)			
	0	1-3	4-6	7,8	Total	0	1-3	4-6	Total
0	17	1	1	0	19	38	1	0	39
1,2	4	15	24	22	65	16	7	2	25
3-6	1	1	7	19	28	5	3	1	9
Total	22	17	32	41	112	59	11	3	73

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Detection of heat shock proteins but not superantigen by isolated oral bacteria from patients with Behcet's disease

T. Miura[1], K. Ishihara[1], T. Kato[1], R. Kimizuka[1], H. Miyabe[1], T. Ando[2], T. Uchiyama[3], K. Okuda[1]

[1]Department of Microbiology, Tokyo Dental College, Chiba, Japan, Departments of [2]Oral Surgery and [3]Microbiology and Immunology, Tokyo Women's Medical University, School of Medicine, Tokyo, Japan

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We isolated bacteria from periodontal sites and mixed saliva in eight patients with Behcet's disease and surveyed them to determine whether they produced heat shock proteins (HSPs) and superantigen. Cultivable bacterial compositions from periodontal sites and saliva were examined by anaerobic culture using blood agar plates. Gram-negative anaerobic rods such as *Prevotella intermedia, Fusobacterium nucleatum*, and *Capnocytophaga* species were predominant in the isolates from the subgingival plaque samples. The *Streptococcus mitis* group was the most common type isolated from the saliva samples. To detect the production of HSPs, Western blot analyses were performed using a polyclonal rabbit antibody to *Escherichia coli* DnaK and a monoclonal antibody to *Helicobacter pylori* Gro-EL. Sonic extracts of 27 of the strains (79.4%) reacted with the antibody against *E. coli* DnaK. Nine of these 34 strains (26.5%) were found to produce HSPs that reacted with antibody to *H. pylori* Gro-EL. A total of 54 isolates were examined for superantigen activity against human peripheral leukocytes. Twenty-five gram-negative clinical strains isolated from chronic periodontitis lesions and 20 ATCC strains of microorganisms were also examined. We could not detect any superantigen activity in 500× diluted supernatant of the strains isolated from the eight patients with Behcet's disease. The present study indicates that the anaerobic strains isolated from the oral cavity of these patients produce HSPs, the production being related to Bechet's disease.

Key words: Behcet's disease; heat shock proteins; oral anaerobe; superantigen

Katsuji Okuda, Department of Microbiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261–8502, Japan
Tel.: +81 43 270 3704;
fax: +81 43 270 3763;
e-mail: kokuda@tdc.ac.jp
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Behcet's disease is an inflammatory disorder of unknown etiology characterized by recurrent oral aphthous ulcers, genital ulcers, uveitis, and skin lesions (14, 27). Microbial infections have been implicated in its development (14, 18). Herpes simplex virus DNA and serum antibodies against the virus have been found in a higher proportion of patients with Behcet's disease than in controls (14). The *Streptococcus mitis* group has been suggested as a causative agent, because these bacteria and the antibodies against them are frequently found in the oral flora and serum, respectively, of patients with the disease (16, 22, 23). However, none of these infectious agents has been proven to cause Behcet's disease.

It is well known that microbial infections can moderate host responses. The production of superantigens and heat shock proteins (HSPs) by infected microorganisms is closely associated with such changing host responses (3, 6, 34, 35, 37, 38). A series of studies has led to the hypothesis that specific antigens including superantigen and HSPs of microorganisms may trigger cross-reactive immunopathologic responses in patients with Behcet's disease (5, 9, 10, 15, 26, 31, 32). In fact, several oral bacterial species including the *S. mitis* group (16, 22, 23), *Prevotella intermedia* (20), *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* (7) have been shown to produce superantigens that stimulate T cells to

proliferate nonspecifically through interaction with class II major histocompatibility complex products. HSPs are highly conserved immunogenic proteins that are often immunodominant antigens produced in bacteria and mammalian cells by a variety of stresses (37, 38). Many research groups have indicated that HSPs have been implicated in the etiology of Behcet's disease (5, 9, 15, 19, 26).

In the present study, we investigated the bacterial composition of the subgingival plaque and saliva collected from patients with Behcet's disease and determined the levels of superantigen and HSP production by the oral bacteria isolated from these patients.

Materials and methods

Subjects

After their informed consent was obtained, eight patients (five men and three women) with Behcet's disease who consulted the Second Hospital of Tokyo Women's Medical University, School of Medicine for periodontal treatment were enrolled in the present study. The mean patient age was 44.4 years (32–61 years). These patients had not received any antibiotics within the previous 4 weeks.

Sampling of dental plaque and saliva

Sites for sampling subgingival dental plaque were isolated with sterilized cotton rolls. Subgingival plaque samples were collected with a sterilized scaler. Mixed saliva samples were also obtained. All samples were immediately transferred into 0.9 ml RTF transport medium (29), diluted in RTF from 10^{-1} to 10^{-7}. Then 100 µl aliquots of each dilution were incubated on Tryptic soy agar plates (BBL Microbiology Systems, Cockeysville, MD) with 10% horse blood, hemin (5.0 µg/ml), and menadione (0.5 µg/ml) and incubated in an anaerobic chamber containing 10% CO_2, 10% H_2, and 80% N_2 for 7 days.

Identification

Colonies from an appropriate number of plates were picked, purified by repeated transfer, and characterized. Colony morphology, gram-staining, cell morphology, motility, and aerobic growth were checked. Biochemical tests included indole production, esculin hydrolysis, nitrate reduction, gelatinase activity, catalase, and fermentation of glucose, lactose, sucrose, cellobiose, and mannitol and the BAPNA test for trypsin-like enzyme described by Loesche (21). The enzymatic activity of the examined strains was also evaluated by the API ZYM system (Bio Merieux S.A. Marcy-L'Etoile, France).

Assay for superantigen production

The cultured supernatants of the isolated strains were examined for the production of superantigen. Twenty ATCC strains of *Actinomyces naeslundii* 15987 and 12104, *Actinomyces israelii* 12102, *Propionibacterium acnes* 11827 and 11828, *A. actinomycetemcomitans* 43718, 33384 and 29523, *P. intermedia* 25611, *Prevotella nigrescens* 33563, *P. gingivalis* 33277 and 53977, *Fusobacterium nucleatum* 25586, *Campylobacter rectus* 33238, *Tannerella forsythia* 43037, *Eikenella corrodens* 23834, and *Treponema denticola* 33521 and 35405, *Mycoplasma penetrans* 15845, and *Mycoplasma buccae* 14851 were also included in this experiment. The Tryptic soy broth culture media for gram-positive and gram-negative bacteria, the TYGVS broth for *T. denticola* strains described in our previous paper (12) and *Mycoplasma* broth (BBL Microbiology Systems) for *Mycoplasma* species were used. Culture supernatants were filtered through 0.1 µm sterile Millex-VV filters (Millipore, Bedford, MA) and evaluated for superantigen activity. The assay for superantigen production was carried out as described by Uchiyama et al. (35). Staphylococcus enterotoxin A (SEA; Toxin Technology, Inc., Miami, FL) was used to stimulate lymphocyte culture as a positive control at a concentration of 400 ng/ml. For assay purposes, the appropriate concentration of SEA as a positive control or broth alone as a negative control was added to the cells. Heparinized venous blood (30–50 ml) from healthy volunteer donors was diluted with phosphate-buffered saline (PBS) and layered over Ficoll–Conray medium containing 100 g of Ficoll 400 medium (Amersham Biosciences, Piscataway, NJ) and 240 ml of Conray 400 medium (66.8% w/v, Daiichi Seiyaku, Tokyo, Japan) per liter, a mixture whose density had previously been adjusted to 1.077 µg/ml with saline. The tubes were centrifuged for 30 min at 500 × g at room temperature. Leukocytes were harvested from the Ficoll–Conray interface and washed twice to remove the Ficoll medium with Hanks' solution (Nissui, Tokyo, Japan) containing 2% fetal calf serum. Cells were collected by centrifugation at 200 × g for 10 min at 4°C, and then resuspended in the RPMI 1640 culture medium with the supplementation of 100 µg/ml streptomycin and 100 IU/ml penicillin. For the superantigen assay, cells were distributed at 1×10^5 viable cells per well in 96-well round-bottomed microplates (Iwaki, Chiba, Japan). After incubation at 37°C in a humidified environment containing 5% CO_2 for 2 days, the cultures were pulsed with methyl-^{3}H thymidine (1 µCi/well) for the last 16 h of incubation, and thymidine incorporation was determined in a liquid scintillation counter. The data are presented as averages of triplicate cultures.

Heat shock protein production of isolates from Behcet's patients

To detect the production of HSPs, Western blot analyses were performed using polyclonal rabbit antibody to *Escherichia coli* DnaK (Hsp 70, Upstate Biotechnology Inc., Waltham, MA) and a monoclonal antibody to *Helicobacter pylori* Gro-EL (Hsp 60, Wako Pure Chemical Industries, Ltd, Osaka, Japan). Cells grown in each medium were harvested by centrifugation and washed twice with PBS (pH 7.2). The cell suspension was homogenized with a sonicator (Branson, Danbury, CT) at 100 W for 5 min on ice, and the supernatants were used in the experiment. These sonic extracts of bacterial strains were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Towbin et al. (33). Separated proteins by SDS-PAGE were transferred to PVDF membranes, and the membranes were then washed twice and incubated with rabbit anti *E. coli* DnaK antibody or mouse anti-*H. pylori* Gro-EL antibody. After washing, the membranes were reacted with peroxidase conjugated goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody. The peroxidase reaction was initiated with Tris buffered saline containing 4 methoxy-1-naphthol and 0.02% H_2O_2.

Results

Bacterial flora of dental plaque and saliva

The bacterial composition of samples obtained from eight patients with Behcet's disease were examined by culture on blood agar plates in an anaerobic chamber. The cultivable compositions of the subgingival plaque and mixed saliva are summarized in Tables 1 and 2, respectively.

The predominant strains from the subgingival plaque samples were groups of *S. mitis* and *Streptococcus salivarius*. Isolated strains of black-pigmented colonies on blood agar in subgingival plaque

Table 1. Comparison (%) of anaerobically cultivable bacteria in samples of subgingival dental plaque obtained from patients with Behcet's disease

	Patients							
Nos.	1	2	3	4	5	6	7	8
Age	37	42	40	51	41	51	32	61
Sex	F	M	M	M	F	M	M	M
Bacterial species in dental plaque	Detection rate (%)							
Streptococcus mitis group	20.2	21.4	9.1	20.1	1.9	–	26.1	17.5
Streptococcus salivarius group	19.0	8.6	12.0	–	–	–	4.3	10.0
Streptococcus mutans group	–	–	–	–	–	–	4.3	–
Actinomyces naeslundii	3.7	10.0	6.0	6.7	–	23.0	13.0	5.0
Actinomyces israelii	–	–	–	–	4.5	–	–	–
Porphyromonas gingivalis	–	–	4.0	20.8	–	–	–	–
Prevotella intermedia	5.1	4.3	6.8	5.4	1.0	–	–	–
Prevotella melaninogenica	–	–	0.9	10.7	22.2	–	8.7	7.5
Prevotella denticola	–	–	–	–	–	3.8	–	–
Prevotella species	–	7.1	6.4	–	–	11.5	–	12.5
Fusobacterium species	8.9	–	1.6	–	–	15.4	–	–
Fusobacterium nucleatum	–	2.8	2.0	7.3	–	7.7	4.3	–
Capnocytophaga species	7.6	7.1	1.5	–	11.2	–	4.3	5.0
Leptotrichia buccalis	–	2.9	2.9	1.0	2.9	7.7	–	–
Eikenella corrodens	–	2.9	–	2.6	–	–	–	10.0
Selenomonas sputigena	–	–	5.0	–	–	–	4.3	–
Veillonella species	1.3	2.9	4.0	7.9	4.0	–	–	–
Unidentified	34.2	30.0	37.8	17.5	52.3	30.9	30.7	32.5

Table 2. Comparison (%) of anaerobically cultivable bacteria in samples of mixed saliva obtained from patients with Behcet's disease

	Patients							
Nos.	1	2	3	4	5	6	7	8
Age	37	42	40	51	41	51	32	61
Sex	F	M	M	M	F	M	M	M
Bacterial species in dental plaque	Detection rate (%)							
Streptococcus mitis group	30.3	25.0	4.7	14.3	10.6	–	77.5	70.0
Streptococcus salivarius group	22.2	21.7	68.1	7.1	9.1	8.8	–	10.0
Veillonella species	–	–	7.1	–	–	–	–	
Actinomyces naeslundii	–	–	–	14.3	18.2	–	–	–
Fusobacterium nucleatum	3.0	–	–	–	–	–	–	10.0
Capnocytophaga species	2.0	–	–	7.1	–	–	–	–
Prevotella intermedia	–	–	–	–	2.9	–	–	
Prevotella species	–	–	4.7	–	–	23.5	3.8	–
Selenomonas sputigena	–	–	–	–	–	–	3.8	–
Unidentified	42.5	53.3	22.5	50.1	62.1	64.8	14.9	10.0

samples from Behcet's patients were identified as *P. intermedia*, *Prevotella melaninogenica* and *P. gingivalis*. However, many of the isolated strains of gram-negative short rods could not be identified by the biochemical characteristics surveyed in this study. More than 10% of the total colony forming units (CFUs) in subgingival samples from patient Nos. 2, 3, 4, 5, 6, and 8 were *Prevotella* species. In addition, we found more than 20% of *P. gingivalis* in the samples obtained from patient No. 5. Genus *Fusobacterium*, including *F. nucleatum* and *Capnocytophaga* species, were also isolated from almost every patient at a moderate detection rate. Most of the isolates in the saliva samples were *Streptococcus* species, mainly groups of *S. mitis* and *S. salivarius*. We could not identify all of the strains isolated from each sample.

Superantigen production

We examined 54 strains isolated from the oral cavities of eight patients with Behcet's disease for their ability to produce superantigen using healthy volunteers' lymphocytes. These 54 strains included 8 strains of the *S. mitis* group, 6 strains of the *S. salivarius* group and 3 strains of the *Streptococcus mutans* group, 4 strains of unidentified *Streptococcus* species, 2 strains of *Veillonella* species, 6 strains of *A. naeslundii*, one strain of *A. israelii*, 2 strains of *P. gingivalis*, 4 strains of *P. intermedia*, 2 strains of *Prevotella denticola* and an unidentified *Prevotella* species, 6 strains of *F. nucleatum*, 4 strains of unidentified *Capnocytophaga* species, 2 strains of *Leptotrichia buccalis*, and one strain each of *E. corrodens* and *Selenomonas sputigena*. The superantigen production was examined using 50× and 500× dilution of the culture supernatant from each culture. The 50× diluted culture supernatant sample from some strains of *Streptococcus* species induced weak thymidine incorporation. However, repeated examinations using 500× diluted culture supernatant sample failed to find superantigen activity in strains isolated from the oral cavities of the eight Behcet's patients.

We also examined the production of superantigen by 25 gram-negative clinical strains isolated from chronic periodontitis lesions and 20 ATCC strains. No superantigen production was found in strains of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *P. nigrescens*, *C. rectus*, *E. corrodens*, *F. nucleatum*, *P. intermedia*, *T. denticola*, *T. forsythia*, *M. buccae* and *M. penetrans*.

Production of HSPs by isolated bacterial strains

The HSPs produced that reacted with the polyclonal antibody against *E. coli* DnaK (HSP 70) and with the monoclonal antibody against *H. pylori* Gro-EL (HSP 60) are summarized in Table 3. The strains examined were 4 strains of *P. intermedia*, 3 strains of *F. nucleatum*, 2 strains each of *S. sputigena* and *L. buccalis, and* one strain each of *P. denticola, P. gingivalis*, and *E. corrodens*. Ten unidentified strains of *Prevotella* species, 5 strains of unidentified *Streptococcus* species, 3 of *Capnocytophaga* species, 1 of *Veillonella* species, and 1 unidentified gram-negative rod were also examined. Twenty-seven strains (79.4%) reacting with anti-*E. coli* DnaK and 9 strains (26.5%) reacting with anti-*H. pylori* Gro-EL were found among 34 strains in the present study. We confirmed these results with repeated Western blot analysis.

Discussion

Oral ulceration is usually an initial symptom and is seen in all patients at some time in the reported clinical course of Behcet's disease (25). To clarify the relationship between the oral ulceration and oral bacterial interactions, including the immunopathologic factors, we attempted to isolate

Table 3. Reactivity of bacterial strains isolated from samples of subgingival plaque and mixed saliva from patients with Behcet's disease against HSPs of *E. coli* DnaK and *H. pylori* Gro-EL

Bacterial species	Number of positive strains/Number of examined strains	
	anti-*E. coli* DnaK	anti-*H. pylori* Gro-EL
Prevotella intermedia	3/4	1/4
Prevotella denticola	0/1	0/1
Unidentified *Prevotella* species	10/10	4/10
Porphyromonas gingivalis	0/1	0/1
Eikenella corrodens	1/1	0/1
Fusobacterium nucleatum	2/3	0/3
Unidentified *Capnocytophaga* species	2/3	1/3
Leptotrichia buccalis	2/2	0/2
Selenomonas sputigena	1/2	2/2
Unidentified *Veillonella* species	1/1	0/1
Unidentified gram-negative rod	1/1	0/1
Unidentified *Streptococcus* species	4/5	1/5
Total	27/34	9/34

and detect superantigen-producing anaerobic bacteria in subgingival dental plaque and saliva from patients with Behcet's disease. We isolated 620 strains from eight patients. Based on gram-staining, cell morphology, biochemical properties, and the results of the anaerobic API enzymatic kit, we identified most of the isolated strains at mainly the genus level. The anaerobic bacterial compositions of the subgingival plaque and saliva are listed in Tables 1 and 2, respectively. We obtained the subgingival plaque from inflammatory gingival sites but for ethical reasons did not record the periodontal status with probes or X-ray photographs. The predominant cultivable bacteria in the subgingival plaque samples were gram-negative short rods. A comparison of the present culture study with cultivable findings of subgingival plaque reported previously by Sutter et al. (30) and Umeda et al. (36) revealed no significant differences. The predominant bacterial composition of mixed saliva in the present study comprised *Streptococcus* species. The present findings are similar to those in a previous cultivable study (24). No specific species or inherent bacterial composition was found in the present study.

Immunomodulatory effects, especially lymphocyte stimulation, are thought to be implicated in the development of Behcet's disease (9, 18, 19, 27, 28). It has been found that *S. mitis* produces superantigen, and an association of extracellular products of oral *Streptococcus* with the pathogenesis of oral mucosal diseases has also been suggested (16, 22, 23). Periodontal disease-associated bacteria such as *P. gingivalis* produce substances with some of the characteristics of superantigens (7), and certain *P. intermedia* have been suggested to activate Vβ-specific T cells in a manner similar to that of other known microbial superantigens (20). We examined the culture supernatants of 54 isolated bacterial strains from eight patients with Behcet's disease, 25 gram-negative strains from chronic periodontitis lesions and 20 ATCC oral bacterial strains in order to detect superantigen, but none of the strains we examined produced any superantigen in the culture supernatant, although 50× diluted sample from some strains of *Streptococcus* species induced weak thymidine incorporation. Further study using concentrated culture supernatant is required to detect the superantigen activity from these strains.

We have previously reported that oral *Mycoplasma* species isolated from HIV-seropositive patients did not produce superantigen (2). It is possible that some culture conditions or stages affect superantigen production. Recent studies have indicated that dental plaque microorganisms form a biofilm with a complex bacterial community that can modulate pathogenic factors (4, 17). In this study, we examined planktonic bacterial cells grown in broth culture. It is possible that subgingival plaque bacteria form biofilms that then produce immunomodulating factors such as superantigen *in vivo*. Further analysis is required to examine the superantigen production of both intact dental plaque and its isolates obtained from patients with Behcet's disease in advancing stages of the disease under various culture conditions.

HSPs have been called 'common antigens' and have been implicated in immunomodulatory actions such as immunosuppression and the induction of autoimmune diseases (37, 38). One immunohistochemical study of HSPs revealed that they are expressed differently in experimental cells of patients with systemic lupus erythematous and atopic dermatitis (8). There are two kinds of responses to HSPs: the response to the infectious pathogen and the recognition of the conserved epitope, which is thought to play some role in autoimmune disease and has therefore been called the 'common antigen'. In addition, T cells with γδTCRs, which are abundant in mucosal membranes, exhibit unusually high reactivity with some HSPs. Oral streptococcus antigens such as HSPs are involved in the etiology of Behcet's syndrome (18, 19, 26, 28). The present study showed that many oral bacterial species isolated from patients with Behcet's disease produce HSP70. We have previously reported that strains isolated from patients with chronic periodontitis with pustulosis palmaris et plantaris (PPP), and HIV-seropositivity, produce HSPs (1, 2, 11). We have also examined the relationships between the onset of PPP, periodontitis and HSP levels of IgG against HSPs, including *A. actinomycetemcomitans* DnaJ, in sera (11). In that study, we found that periodontal therapy and extraction of teeth with periapical infection resulted in remission of PPP and a statistically significant reduction in the levels of IgG against HSPs.

In the present study, the mean ratios of HSP-producing bacterial strains that reacted with both antibodies were significantly lower than those in the strains isolated from patients with Behcet's disease we examined previously. We previously found that *C. rectus* strains isolated from adult periodontitis lesions possessed cross-reacting antigens including HSP cross-reactive with *H. pylori* strains (13). As infectious disease progress, it is possible that immune responses to HSPs may be initiated, and it is also possible that the differences we note may be related to immune suppression. The results of the present study do not provide sufficient data to allow discussion of the relationship between bacterial infections in the oral cavity and the progression of Behcet's disease. However, it is still possible that HSPs produced by microorganisms in the oral cavity may lead to the acceleration of oral membrane ulceration in patients with Behcet's disease.

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Salivary bisphenol-A levels detected by ELISA after restoration with composite resin

N. SASAKI, K. OKUDA, T. KATO*
Department of Microbiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba-shi, Chiba 261-8502, Japan
E-mail: tekato@tdc.ac.jp

H. KAKISHIMA, H. OKUMA, K. ABE
Eiken Chemical Co., Ltd., 1381-3 Shomoishigami, Ohtawara, Tochigi 324-0036, Japan

H. TACHINO, K. TUCHIDA, K. KUBONO
SRL, Inc., 2-41-19 Akebono-cho, Tachikawa-shi, Tokyo 190-8567 Japan

Bisphenol-A diglycidylether methacrylate (Bis-GMA), which is synthesized from bisphenol-A (BPA), a compound with exogenous endocrine disrupter action, is widely used as a dental material. During clinical filling with sealants and composite resins, the compounds are solidified by polymerization and then used. However, it has been noted that unpolymerized monomers may become dissolved in saliva. In this study using a competitive ELISA system, we investigated the changes in the BPA concentration in saliva after restoration with composite resins. Commercial composite resins from nine companies were tested. Mixed saliva was collected from 21 subjects. Based on the dynamics of salivary BPA detected by this ELISA system, we concluded that several tens to 100 ng/ml of BPA were contained in saliva after filling teeth with composite resin but that sufficient gargling can remove it from the oral cavity. Our data suggest that sufficient gargling after treatment is important for risk management.

1. Introduction

Bisphenol-A (BPA) is a major component of epoxy resin and polycarbonate resin, and it has been suspected in recent years of having endocrine disrupter action as an environmental hormone. It has been reported that 2.3–3.6 ng/ml of BPA is generated in the air [1] and that its influence on the water environment and ecosystem is of concern [2, 3]. Regarding the effects of BPA on animals, vom Saal *et al.* [4] reported that administration of 1/25 of the acceptable daily intake, 2 μg/kg body weight, to pregnant mice induced hypertrophy of the prostate in newborn males, and Takai *et al.* [5] reported that growth of early mouse embryos was promoted.

BPA is widely used as a starting material for sealants and composite resins worldwide. In clinical filling with sealants and composite resins, the compounds are solidified by polymerization and then used. However, it has been noted that unpolymerized monomers may be dissolved in saliva and thus the patient may be exposed to the monomer [6–11]. Recent improvements of instrumental performance have allowed more precise analytical results than those obtained by the previous analytical methods. However, such analyses were performed using liquid chromatography (HPLC), gas chromatography/mass spectrometry (GC/MS), and UV [12], requiring complex pretreatments, and these methods are not suitable for the treatment of a considerable number of samples. Using an enzyme-linked immunosorbent assay (ELISA) system that readily measures serum and plasma BPA without pretreatment by extraction, BPA in saliva samples was measured before and after restoration with various composite resins.

2. Materials

Nine commercially available products were used in this study: A: Z 100 (3 M, St. Paul, MN); B: Progress (Kanebo, Ltd., Tokyo, Japan); C: Palfique Toughwell (Tokuyama Corp., Tokyo, Japan); D: Matafil Flo (Sun Medical Co., Ltd., Shiga, Japan); E: Unifil S (GC Corp., Aichi, Japan); F: Beautifil (Shofu Inc., Kyoto, Japan); G: Xeno CFII (Sankin Kogyo, Tochigi, Japan); H: Prodigy (Kerr Corp., Orange, CA); and I: Cleafil ST (Kuraray Co., Ltd., Okayama, Japan) (Table).

3. Methods

3.1. Method of restoration with composite resin

After informed consent was obtained, 21 patients underwent cavity preparation. All of the method in this

*Author to whom all correspondence should be addressed.

study followed "The Guidelines for Human Studies". Almost the same size of cavity was used in each treatment. The bonding agent provided by each maker was applied to a prepared cavity, which was then irradiated with visible radiation for 30 s. according to the manufacturer's recommended procedure. Each cavity was then filled with 0.1 g of composite resin, irradiated with visible radiation for one min, and ground with a silicon point for polishing.

3.2. Collection of saliva samples

Saliva was collected for five minutes while the subject bit a paraffin pellet used in saliva sampling for oral bacterial testing. Each specimen was centrifuged at 3,000 rpm for 10 min, and the supernatant was used. Samples were collected before filling, immediately after filling with composite resin, and after gargling with tepid water at about 37 °C for 30 s. All samples were analyzed after incubation at 4 °C for 24 h in glass tubes. Aloka RIA Program ARCAS (Aloka Co., Ltd., Tokyo, Japan) was used for data management. In one patient treated with composite resin-A (A-4), BPA in saliva was measured over 120 h after gargling as described above.

3.3. Measurement of BPA

BPA was measured using the BPA ELISA 'EIKEN' Kit (Eiken Chemical Co. Ltd., Tokyo, Japan). This method is a competitive enzyme linked immunosorbent assay (ELISA) developed by Ohkuma *et al.* [13] that measures BPA in biological specimens such as serum and plasma. To the secondary antibody-coated microplates, 20 μl of the standard BPA or sample, 50 μl of enzyme-labeled antigen [horseradish peroxidase labeled BPA-4-carboxyphenolether (CPhE)], and 50 μl of anti-BPA serum were added and reacted at room temperature for one hour. After the reaction solution was removed, 300 μl of washing solution was added to each well and removed. After this procedure was repeated three times, 100 μl of o-phenylenediamine (OPD) solution was added. After the enzyme reaction proceeded at room temperature for 30 min, the reaction was stopped by adding 100 μl of 2 N sulfuric acid, and the optical density (OD) was measured at 492 nm.

4. Results

The composite resins used in this study are summarized in the Table I. The composite resins in A, C, F, G, H, and I are Bis-GMA monomer-based composite resins and bonding agents, and those in B, D, and E are urethane dimethacrylate (UDMA) monomer-based composite resins and bonding agents. In the four patients treated with composite resin-A, the salivary BPA levels before and after restoration and after gargling were 0.3–2.0 ng/ml (mean ± standard deviation: 0.87 ± 0.69 ng/ml), 21.0–60.1 ng/ml (32.1 ± 16.27 ng/ml), and 1.6–4.7 ng/ml (3.1 ± 1.47 ng/ml), respectively (Fig. 1). In one (A-4) of the above patients, saliva was collected for five days after gargling by the same procedure, and BPA was measured. The BPA level varied within the range from that before restoration to that after gargling within a half day; then it converged to the level before restoration (Fig. 2). When we tested C, F, G, H and I, which are made of the same Bis-GMA as A, the BPA level was low even immediately after restoration in those patients treated with G and I. In contrast, in three patients treated with F, the BPA level tended to be high, and a higher level was also present even after gargling (Fig. 1). In patients treated with the non-Bis-GMA materials, the BPA concentration was 40 ng/ml or lower, even in the patient with the highest level immediately after restoration (D-1); these data indicate that the level was generally low (Fig. 3).

TABLE I Composite resins and bonding agents used in this study

	Restoratives (Manufacturers)	Source monomer	Bonding agent
A	Z 100 (3 M)	Bis-GMA/TEGDMA	Bis-GMA
B	Progress (Kanebo)	UDMA/TEGDMA	UDMA/TEGDMA
C	Palfique Toughwell (Tokuyama)	Bis-GMA	Bis-GMA
D	Metafil Flo (Sun Medical)	UDMA/TEGDMA	UDMA
E	Unifil S (GC)	UDMA	UDMA
F	Beautifil (Shofu)	Bis-GMA/TEGDMA	Bis-GMA
G	Xeno CFII (Sankin Kogyo)	Bis-GMA	Bis-GMA
H	Prodigy (Kerr)	Bis-GMA/TEGDMA	Bis-GMA
I	Cleafil ST (Kuraray)	Bis-GMA/TEGDMA	Bis-GMA

Bis-GMA: Bisphenol-A diglycidylether methacrylate.
TEGDMA: Triethylen glycol dimethacrylate.
UDMA: Urethane dimethacrylate.

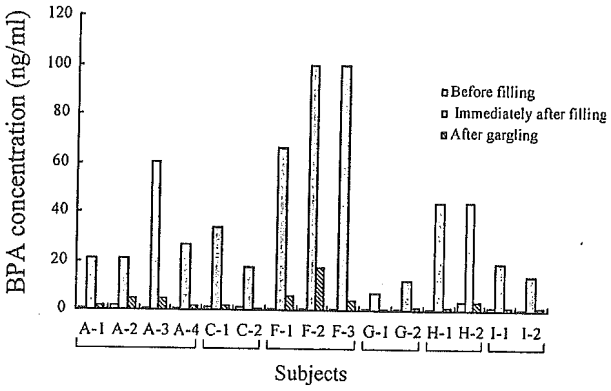

Figure 1 Salivary BPA concentrations before and after restoration with Bis-GMA- and TEGDMA-based composite resins.

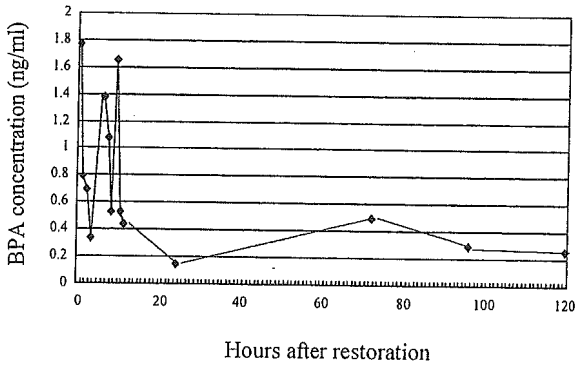

Figure 2 Time course of BPA concentration after restoration with composite resin-A, which is mainly composed of Bis-GMA.

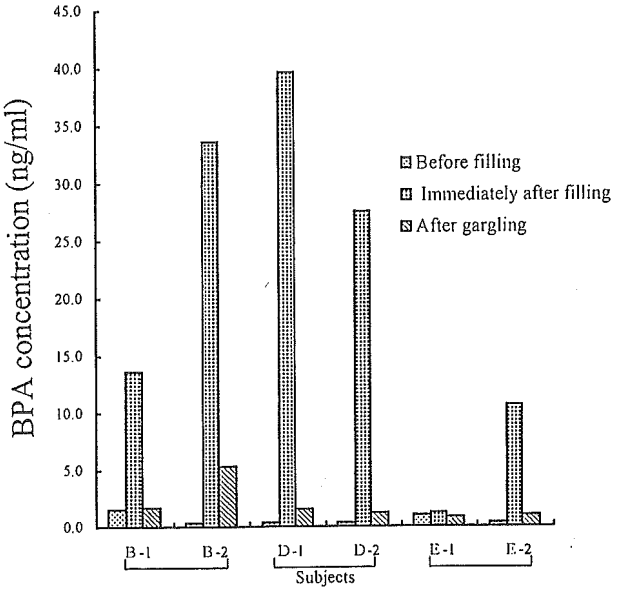

Figure 3 Salivary BPA concentrations after restoration with urethane monomer-based composite resin

5. Discussion

Olea *et al.* [6] initially reported the problem of dissolution of BPA. The amount of BPA dissolution from one resin-based sealant used in their study was abnormally high, and this product contained Bis-DMA. Many researchers have performed additional studies [7, 8, 14–17]. Recently, Tarumi *et al.* [18] analyzed the BPA content in three types of sealant and five types of bonding material by HPLC equipped with a UV detector and evaluated the estrogen activity in these materials by a receptor gene assay using Hela cells. They tested three types of sealant but did not detect BPA in any of the products. However, estrogen activity was detected in two products containing Bis-DMA, and they concluded that this activity was due to Bis-DMA, not due to BPA. Ohsaki and Imai [19] performed tissue analysis of commercial Bis-GMA, and confirmed that in addition to Bis-GMA and its structural isomer, iso-Bis-GMA, 2,2 [4-(2-hydroxy-3-metacryloiroxy-1-propoxy)-4-(2,3-dihydroxy-1-propoxy)] diphenylpropane (Bis-GMA-H), which possesses a structure in which one metacrylate ester bond of Bis-GMA is hydrolyzed, was present. They pointed that this Bis-GMA-H may appear as a peak overlapping the BPA peak under certain analytical HPLC conditions. The high BPA content reported by Olea *et al.* [6] might be due to inappropriate separation conditions.

Because BPA may dissolve from the inner coating materials of canned food, the subjects were instructed to avoid drinking canned beverages from one week before their dental treatment in our study. As in the study reported by Noda *et al.* [12], dissolution of BPA from non-Bis-GMA composite resins was detected in this study. Dissolution of BPA from non-Bis-GMA suggests slight contamination during the synthesis process. However, the level after gargling was very low. The amount of BPA in saliva collected before treatment was equal to that in the umbilical cord, and this detection in saliva suggested contamination *via* pathways other than dental treatment.

Not only Bis-GMA but also many BPA analogues are used as monomers in sealants, composite resins, and bonding agents for dental treatment [19]. Based on the published range, these dental materials are not composed of a single compound, and removal of simple unpolymerized compounds, byproducts, and impurities is difficult at the time of polymerization from a practical standpoint. This fact makes any discussion of safety confirmation of materials concerned in endocrine disrupter action complex. Is BPA released? What level is the detection level? It is undeniable that separation analysis by HPLC and GC/MS is too complex for frequent analysis near clinical practice [12, 14, 15, 17, 20, 21]. Although the values measured using this ELISA system may include a large amount of crossed compounds among impurities and polymerization byproducts contained in dental materials composed of multiple ingredients, because the cross-reactivity among Bis-DMA, TEGDMA, and HEMA contained in monomers is low, only salivary BPA may have been detected [13]. Based on the dynamics of salivary BPA detected by this ELISA system, we conclude that several tens to 100 ng/ml BPA were present in saliva after filling cavities with composite resin but that sufficient gargling can remove this compound from the oral cavity. After removal by gargling, the BPA concentration converged to a constant level after half a day. Depending on the restorative material, the concentration can be reduced to a level lower than 10 ng/ml even immediately after restoration. Therefore, for dental treatment of pregnant women and children, who are readily affected by endocrine disrupters, it is important to insist upon sufficient gargling after treatment and/or to select materials with consideration of risk management.

It has been reported that BPA is degraded slowly by gram-negative aerobic rods [22], and possible conversion to stilbene during the bacterial degradation process is being clarified. The questions of how oral bacteria degrade BPA dissolved in saliva, how they convert BPA to stilbene, and which species of bacteria are involved in this process remain for future clarification.

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RESEARCH ARTICLE

Prime-boost vaccination with plasmid DNA and a chimeric adenovirus type 5 vector with type 35 fiber induces protective immunity against HIV

K-Q Xin[1], N Jounai[1], K Someya[2], K Honma[3], H Mizuguchi[4], S Naganawa[5], K Kitamura[5], T Hayakawa[4], S Saha[1], F Takeshita[1], K Okuda[3], M Honda[2], DM Klinman[6] and K Okuda[1]

[1]Department of Molecular Biodefense Research, Yokohama City University, Graduate School of Medicine, Yokohama, Japan; [2]AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan; [3]Department of Microbiology, Tokyo Dental College, Chiba, Japan; [4]Laboratory of Gene Transfer and Regulation National Institute of Biomedical Innovation, Osaka, Japan; [5]Department of Public Health, Yokohama City University, Graduate School of Medicine, Yokohama, Japan; and [6]Center for Biologics Evaluation and Research, US Food and Drug Administration, Bethesda, MD, USA

Immunization involving a DNA vaccine prime followed by an adenovirus type 5 (Ad5) boost elicited a protective immune response against SHIV challenge in monkeys. However, the hepatocellular tropism of Ad5 limits the safety of this viral vector. This study examines the safety and immunogenicity of a replication-defective chimeric Ad5 vector with the Ad35 fiber (Ad5/35) in BALB/c mice and rhesus monkeys. This novel Ad5/35 vector showed minimal hepatotoxicity after intramuscular administration with the novel Ad5/35 vector. In addition, an Ad5/35 vector expressing HIV Env gp160 protein (Ad5/35-HIV) generated strong HIV-specific immune responses in both animal models. Priming with a DNA vaccine followed by Ad5/35-HIV boosting yielded protection against a gp160-expressing vaccinia virus challenge in BALB/c mice. The Ad5/35-HIV vector was significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector. These findings indicate that an Ad5/35 vector-based HIV vaccine may be of considerable value for clinical use.
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Introduction

A vaccine capable of preventing HIV infection is needed to control the global AIDS pandemic. In the past decade, multiple strategies to produce an immunogenic HIV vaccine have been explored. This included production of HIV subunit peptide vaccines,[1] DNA vaccines,[2] recombinant virus-vector vaccines (including modified vaccinia virus,[3] adenovirus (Ad),[4,5] rabies virus,[6] flavivirus,[7] sendai virus,[8] Venezuelan equine encephalitis virus,[9] and adeno-associated virus[10,11]), and bacterial vector-vaccines (bacille Calmette–Guerin,[12,13] and *Lactococcus lactis*[14]). Each of these strategies showed some promising results in animal models, either alone or in combination.

Among these vectors, the replication-defective human Ad type 5 (Ad5) recombinants (with the deletion of a replication-essential gene, E1) and the replication-defective modified vaccinia Ankara (MVA) elicited the most potent $CD8^+$ T-cell responses and provided the highest degree of protection in non-human primates.[3,4,15,16] A major limitation for the clinical application of the Ad5 and MVA vectors is the pre-existing immunity against these viruses in humans, since most of the human population has been infected with Ad5[17] and vaccinia virus on being administered the smallpox vaccine. The pre-existing antiviral immunity may strongly influence the efficacy of the HIV vaccine using Ad5 and MVA vectors.

Human Ads are classified into six subgroups from A–F.[18] Most of Ad serotypes belonging to subgroups A, C, D, E, and F use the coxsackievirus and adenovirus receptor (CAR) as a cellular receptor.[19] The Ad5 (subgroup C) has well-defined biological properties and has been widely used as a vector for gene therapy and vaccine. The replication-defective Ad5 vector can easily be produced in high titers and is highly effective in boosting HIV-specific immunity.[4,15] However, this virus uses CAR as its primary attachment receptor, which confers tropism for liver parenchymal cells.[19–22] This raises important safety concerns,[22] particularly because the administration of an Ad5-based vector for gene therapy resulted in the death of a patient.[23] In response to these shortcomings, our laboratory has examined the immunogenicity and safety of a replication-defective chimeric Ad5 vector with Ad type 35 fiber (Ad5/35) (Ad35 virus was classified as subgroup B). The Ad35 fiber showed 25% amino-acid homology with the Ad5 fiber.[24] Cell entry of Ad35 is CAR independent and may involve CD46 receptor, which expresses on most human cells.[25] Ad35 can be transducted to liver nonparenchymal cells on a level 4–5 log orders lower than Ad5, but not to

Correspondence: Dr K Okuda, Department of Molecular Biodefense Research, Yokohama City University, Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
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liver parenchymal cells.[20] In the present study, we found that the Ad5/35 recombinants not only induced strong antigen-specific humoral and cellular immune responses and exhibited minimal hepatotoxicity in both mice and non-human primates, but were also significantly less susceptible to the pre-existing Ad5 immunity than a comparable Ad5 vector.

Results

Biodistribution of Ad in mice

In the initial experiments, mice were injected intramuscularly (i.m.) with 10^{11} viral particles (vp) of a luciferase-expressing Ad5 (Ad5-Luc) or Ad5/35 vector (Ad5/35-Luc). Luciferase expression was monitored using an *in vivo* imaging system (IVIS) on days 3 and 10 after administration. As shown in Figure 1a, all of the Ad5/35-Luc vector remained at the injection site. In contrast, substantial amounts of the Ad5 vector migrated to the liver. This difference in vector distribution was confirmed by studies involving LacZ-expressing Ad5 and Ad5/35 vectors (data not shown). Studies on serum glutamic-oxaloacetic transaminase (GOT) and serum glutamic-pyruvic transaminase (GPT) levels revealed that mice injected with the Ad5-Luc vector had changes indicative of liver damage (Figure 1b). We also analyzed serum levels of key proinflammatory cytokines (IFN-γ and IL-6) on days 0, 3, and 10 after administration of virus vectors. The levels of IFN-γ and IL-6 were significantly elevated following administration of Ad5-Luc vector, but not of Ad5/35-Luc vector (Figure 1c). Thus, the hepatotoxicity caused by the Ad5 vector was circumvented by the use of an Ad5/35 vector.

Time-course study of HIV-specific immune responses in mice. Ad5/35 vector can efficiently transfect antigen-presenting cells[18,21,26,27] and muscular cells (Figure 1a). In order to explore whether the virus vector can be used as a vaccine vector, we constructed an HIV Env gp160-expressing Ad5/35 vector (Ad5/35-HIV). The expression of HIV gp160 was confirmed by Western blotting (Figure 2a). The HIV Env gp160-expressing DNA vaccine (DNA-HIV) used in this study was reported previously.[28] The mice were immunized with 10^{10} vp of Ad5/35-HIV vector, and the HIV-specific cellular immune response was periodically monitored by the intracellular cytokine staining (ICS) assay. The assay has been widely utilized to distinguish the relative contributions of $CD8^+$ cells to the overall T-cell responses.[29] On day 3, HIV-specific IFN-γ-secreting $CD8^+$ T cells can be detected (Figure 2b) and peaked 2 weeks after immunization. On day 50 and month 7 after final immunization, 2.5 and 1.2% of HIV-specific IFN-γ-secreting $CD8^+$ T cells still persisted, respectively.

Mice were vaccinated with Ad5/35-HIV vector to explore the humoral immune response 7 weeks after the final immunization. The animals immunized with 10^{10} vp of Ad5/35-HIV vector developed a high-tittered anti-gp160 antibody (Ab) response (Figure 2c). The specificity of the Ab response was confirmed by Western blotting (Figure 2c, upper panel). The magnitude of this response was not significantly altered by preimmunization with the DNA-HIV vaccine (Figure 2c). DNA-HIV vaccination alone generated a low level of HIV-specific serum Ab (Figure 2c, bottom panel). HIV-specific neutralizing Ab was only detectable in the Ad5/35-HIV

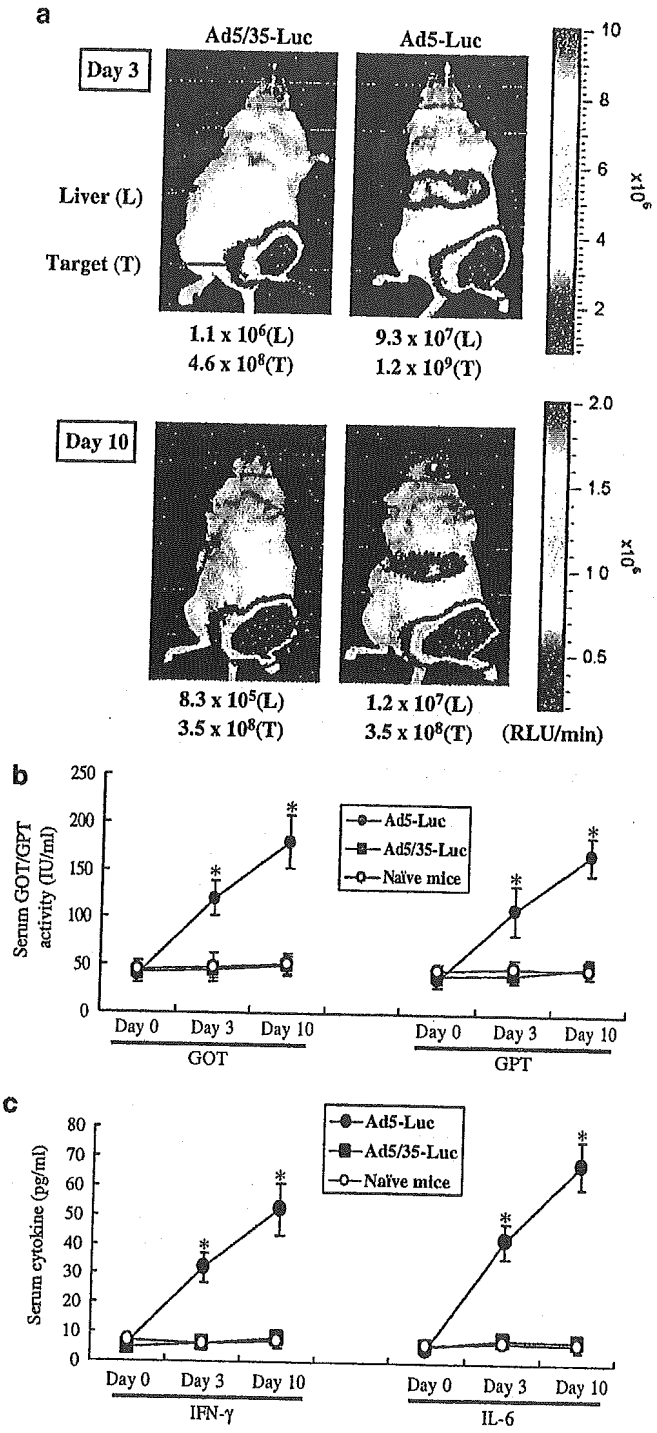

*Figure 1 Biodistribution and safety of Ad vectors. BALB/c mice were injected i.m. with 10^{11} vp of the Ad5-Luc or Ad5/35-Luc vector. (a) Using an IVIS CCD camera, vector distribution was detected after the addition of luciferin (3 mice/group) (expressed in relative light units (RLU)). One of the mice is represented and other mice used show the same pattern. (b) Serum GOP and GPT levels were measured on days 0, 3, and 10 after injection (5 mice/group). IU: international unit. (c) Serum IFN-γ and IL-6 levels were measured on days 0, 3, and 10 after injection (5 mice/group). *Mean values are significantly different between Ad5-Luc-administered mice and Ad5/35-Luc-administered mice or naïve mice at the same time point.*