urinary markers may also be appropriate. Many studies [9–13] have reported the efficacy of serum and urinary markers to evaluate general bone metabolism.

Furthermore, when we evaluate the jawbone, standardization of criteria is an important issue. In the past, several investigations were conducted using mandibular inferior cortex (MIC) classification according to the method of Klemetti et al. to evaluate the jawbone [14–18]. This classification is an assessment of the morphology of the inferior cortex of the mandible on dental panoramic radiographs. Radiographic findings are affected by many factors, i.e., difficulties in standardizing head position, X-ray projection, radiation dose, and anatomic variability of bony structures. However, it was reported that disagreement caused by positioning error and operator error for the MIC was negligible [19]. The MIC is a visible bony structure in the jawbone [14]. Some investigators have reported satisfactory levels of reproducibility of the MIC classification [14–18].

The purpose of this study was to investigate whether a link exists between the jawbone measured by MIC classification and general bone metabolism, as assessed by serum and urinary markers, in community-dwelling older adults.

Materials and methods

Subjects and measurements

We targeted 200 elderly (screened population) subjects in Niigata City, Japan who have participated in an annual examination since 1998. They filled out a detailed questionnaire, including daily activities, nutritive intake, medical history, and smoking habit. Twenty-one people withdrew because they did not consent to this study. Seventeen people were excluded because of a diagnosis of fracture from X-ray assessment by medical doctors: arm (n=2), leg (n=5), spine (n=10). The fractures at the thoracic and lumbar spine were assessed on lateral spine radiographs using semi-quantitative assessment [20]. Twenty-eight people who had taken the following medicine that might affect bone metabolism were also excluded: tamoxifen (n=2), bisphosphonate (n=7), anabolic steroid (n=1), estrogen (n=18).

Out of 200 people, 134 people (study subjects) agreed to additional laboratory tests in 2005. At the time of this survey, all subjects were 77 years old. All subjects were Japanese and did not require special care for their daily activities. The protocol was reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Niigata University.

For study subjects (n=134), blood samples were taken in the morning (0900 to 1100), and serum samples were obtained by centrifugation. When serum samples were collected, the usual food and fluid intake was encouraged. Second morning void urine samples were collected after an overnight fast. The serum and urine samples were stored frozen at −60°C until assayed.

The value of serum bone-specific alkaline phosphatase (S-BAP) was used as a bone formation marker; urinary N-telopeptide cross-links of type I collagen (U-NTX) was used as a bone resorption marker. All laboratory tests were done at a commercial laboratory (BML Inc., Tokyo, Japan) as follows. S-BAP was quantified by an enzyme immunoassay (EIA) (OSTEOLINKS-BAP; Metra Biosystems, Inc., Mountain View, CA, USA). The intra- and interassay coefficient of variations (CVs) in the BML laboratory were less than 3% for S-BAP. U-NTX was quantified by an enzyme-linked immuno-sorbent assay (ELISA) (Osteomark; Ostex International, Inc., Seattle, WA, USA). U-NTX data were corrected by the urinary creatinine (Cr) concentration measured by a standard colorimetric method. The intra- and interassay CVs in the BML laboratory were less than 5% for U-NTX.

All dental panoramic radiographs were obtained using SUPER VERAVIEW X-500 (Morita Co., Tokyo, Japan) at 5–10 mA and 15 s; the kV varied between 60 and 80. We used screens of speed group 200 (HG-M; Fuji Photo Film Co., Tokyo, Japan) and film (UR-2; Fuji Photo Film Co., Tokyo, Japan). To evaluate the jawbone, we used the MIC classification. The inferior cortex was detected on both sides of the mandible, distally from the mental foramen (Fig. 1). Subjects were divided into three groups (C1–C3), according to the following criteria: C1) the endosteal margin of the cortex was even and sharp on both sides; C2) the endosteal margin showed semilunar defects (lacunar resorption) or seemed to form endosteal cortical residues (one to three layers) on one or both sides; C3) the cortical residues were clearly porous. Dental panoramic radiographic measurement was estimated by one of authors. The observer had 5 years' experience of using the MIC classification. The κ values for intra- and interobserver agreement were 0.76–0.85 and 0.72–0.79, respectively.

The subjects' height and weight were measured to the nearest 1 mm or 0.1 kg, respectively, to calculate the body mass index (kg/m^2, BMI). We also utilized data on bone stiffness, which was measured using an ultrasound bone densitometer (AOS-100NW; ALOKA, Co., Tokyo, Japan). The ultrasound signal was sent to the os calcis. Three parameters were determined: transmission index (TI), speed of sound (SOS, m/s), and stiffness. Stiffness was calculated by the software of the device from the values of TI and SOS measurements (stiffness = TI × SOS^2). Stiffness was indicated on the monitor of the device as a percentage of the value of the normal younger generation. The machine was re-calibrated per established protocol each time it was moved. The CV of ultrasound densitometry was less than

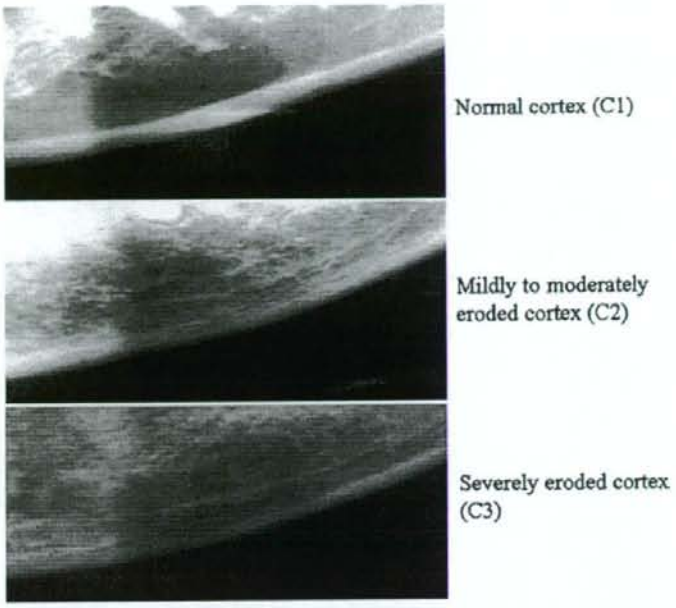

Fig. 1 Mandibular inferior cortex (MIC) classification on dental panoramic radiographs

2%. Ultrasound densitometry enables measurement of the physical properties of bone [21–23].

Data analysis

After 134 subjects were divided into three groups by MIC classification, we evaluated the relationship between S-BAP or U-NTX and MIC classification by Scheffe's multiple comparison test. Multiple linear regression analyses were performed to assess the relationship between S-BAP or U-NTX and MIC classification. S-BAP or U-NTX was used as a dependent variable. MIC classification (C1: 0, C2 or C3: 1), gender (men: 0, women: 1) stiffness, BMI, smoking habit (non-smoker: 0, smoker: 1) were selected as independent variables.

The level of significance was set at $p<0.05$ for these tests. The statistical analyses were carried out using STATA statistical software package (Stata 7.0 for Windows, Stata Corporation, College Station, TX, USA).

Results

Table 1 shows the characteristics of subjects in this study. The percentages of C1, C2 and C3 subjects were 66.2%, 31.1% and 2.7% for men and 16.7%, 45.0% and 38.3% for women, respectively. The percentage of subjects with C2 and C3 was significantly higher in women than men (Fisher's exact probability test, $p<0.001$).

The mean values of S-BAP were 22.4±6.2 U/L for C1, 27.9±10.2 U/L for C2 and 29.7±10.8 U/L for C3 (Fig. 2). A significant correlation was found between MIC classification and S-BAP by Scheffe's multiple comparison test (C1 vs. C2: $p<0.01$, C1 vs. C3: $p<0.01$, C2 vs. C3: NS). The mean

Table 1 Characteristics of subjects in this study

	Normal cortex (C1)	Mildly to moderately eroded cortex (C2)	Severely eroded cortex (C3)
Number of subjects[a]			
Men	49	23	2
Women	10	27	23
Stiffness*[b] (%)	89.5±12.3	88.0±11.9	82.1±6.5
Body mass index*[c] (kg/m^2)	22.0±2.6	23.5±3.4	23.6±4.0
Number of smokers [d]			
Men	13	4	0
Women	0	0	0

*mean ± SD

[a] The study subjects (n=134) were divided into three groups (C1–C3) by mandibular inferior cortex (MIC) classification. The percentage of subjects with C2 and C3 was significantly higher in women than men (Fisher's exact probability test, $p<0.001$)

[b] Stiffness in C3 was significantly lower than in C1 ($p<0.05$) by Scheffe's multiple comparison test

[c] There was no significant difference in BMI among C1, C2 and C3

[d] The percentage of male smokers was significantly higher than female smokers (Fisher's exact probability test, $p<0.001$)

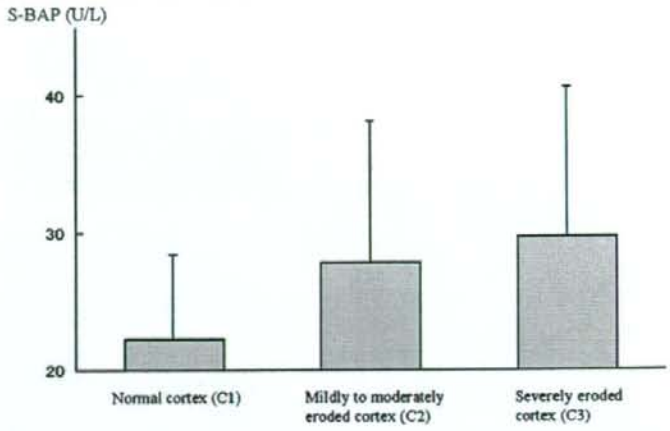

Fig. 2 The relationship between mandibular inferior cortex (MIC) classification and serum bone-specific alkaline phosphatase (S-BAP). C1 vs. C2: $p<0.01$, C1 vs. C3: $p<0.01$, C2 vs. C3: NS

values of U-NTX were 29.0±10.8 nM BCE/mM Cr for C1, 39.9±16.9 nM BCE/mM Cr for C2 and 52.2±20.3 nM BCE/mM Cr for C3 (Fig. 3). A significant correlation was found between MIC classification and U-NTX by Scheffe's multiple comparison test (C1 vs. C2: $p<0.01$, C1 vs. C3: $p<0.001$, C2 vs. C3: $p<0.01$). A significant correlation was found between S-BAP and U-NTX by Pearson's correlation coefficient ($r = 0.65$, $p<0.0001$).

Table 2 presents the result of multiple linear regression analysis using S-BAP as a dependent variable. The MIC classification was significantly associated with the value of S-BAP (standardized coefficient = 0.243, $p<0.05$; coefficient of determination = 0.154, $p<0.001$) after controlling for gender, stiffness, BMI, smoking habit. Table 3 presents the result of multiple linear regression analysis using U-NTX as a dependent variable. The MIC classification was significantly associated with the value of U-NTX (standardized coefficient = 0.226, $p<0.01$; coefficient of determination = 0.394, $p<0.0001$) after controlling for the confounding factors.

Discussion

Our study is the first to demonstrate the association between dental panoramic radiographic findings and biochemical markers of bone turnover in both older (77 years old) men and women. The results of our study indicate that the mandibular inferior cortical erosion finding is significantly associated with increased S-BAP and U-NTX levels. It was suggested that the mandibular inferior cortical finding on dental panoramic radiographs was associated with BMD of the mandibular cortex [24]. Higher S-BAP and U-NTX levels indicate higher bone turnover [9, 12]. Therefore, we suggest that there is an association between the mandibular inferior cortex and general bone metabolism.

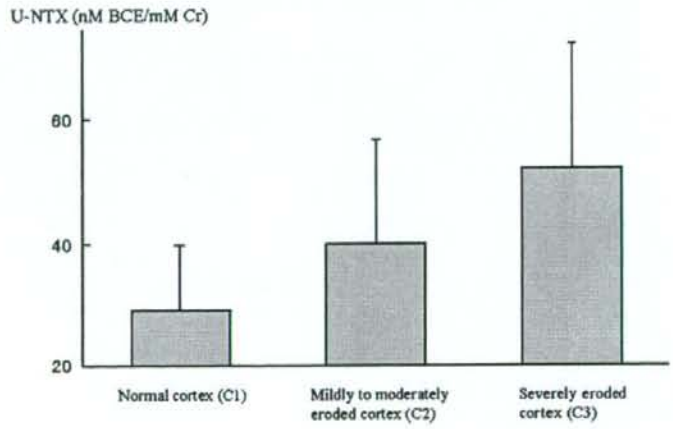

Fig. 3 The relationship between mandibular inferior cortex (MIC) classification and urinary N-telopeptide cross-links of type I collagen (U-NTX). C1 vs. C2: $p<0.01$, C1 vs. C3: $p<0.001$, C2 vs. C3: $p<0.01$

Table 2 The result of multiple linear regression analysis using S-BAP* as a dependent variable

S-BAP (U/L)	Standardized coefficient	*p*
MIC classification ** (C1: 0, C2 or C3: 1)***	0.243	<0.05
Gender (men: 0, women: 1)	0.197	NS
Stiffness (%)	−0.059	NS
Body mass index (kg/m^2)	0.047	NS
Smoking habit (non-smoker: 0, smoker: 1)	0.119	NS
Constant		<0.01

Number of subjects=134
Coefficient of determination (R^2)=0.154 (p<0.001)
* Serum bone-specific alkaline phosphatase
** Mandibular inferior cortex classification
*** Normal cortex (C1), mildly to moderately eroded cortex (C2), severely eroded cortex (C3)

We used both the bone formation marker (S-BAP) and the bone resorption marker (U-NTX) to evaluate bone condition. In particular, the difference of U-NTX among the three groups (C1–C3) was remarkable. In the Japanese population, U-NTX would be the most sensitive predictor of bone loss [12], and people with more than 35.3 nM BCE/mM Cr are considered to have disrupted regulation of bone metabolism [25]. U-NTX of C2 and C3 was higher than the standard level.

In our study, MIC classification correlates more with U-NTX than S-BAP. S-BAP is an enzymatic activity of osteoblastic cells [26]. U-NTX is a marker of fractions of total bone-derived pyridinolines [27]. U-NTX is likely to reflect a different stage of bone turnover compared with S-BAP. However, there are few investigations about relationship between dental panoramic radiographic findings and biochemical markers of bone turnover, although one previous study [28] regarding them in postmenopausal women was performed. The cause of this result in our study is not clear, and further studies are needed.

Since a higher biochemical marker of bone turnover is one of the predictors of future bone loss [10, 12] or osteoporotic fractures [9, 11], the mandibular inferior cortical finding may be useful for identifying older individuals with an undetected increased risk of them. During past decades, some investigators have demonstrated the usefulness of dental panoramic radiographs to evaluate bone condition. Subjects with an abnormal view of the mandibular cortex on dental panoramic radiographs had significantly lower skeletal BMD [14–18]. The mandibular inferior cortical finding on dental panoramic radiographs in older people was strongly associated with a report of osteoporotic fractures [29]. From the above-mentioned studies and our findings, we can expect the clinical application of screening for osteoporosis.

Because dental panoramic radiographs can be taken as part of general dental practice [30–33], the mandibular inferior cortical finding on such radiographs may provide dentists with a mean to identify patients with undetected low skeletal BMD or at high risk of suffering osteoporotic fractures. It is possible that dentists would be able to refer these patients to medical professionals for further examinations.

We also found a significant association between MIC classification and stiffness (C1 vs. C3: p<0.05) (Table 1). People with lower % of stiffness measured by AOS-100NW were considered to be high risk group of fracture [23]. Unlike quantitative computed tomography (QCT) and dual energy X-ray absorptiometry (DXA), quantitative ultrasound of bone (QUS) is a radiation-free method, relatively inexpensive, and easily transportable. Therefore, QUS devices have been proposed as screening tools for osteoporosis and/or fracture risk assessment [21, 22]. Furthermore, stiffness was significantly associated with the value of U-NTX (Table 3). There would be relationships among MIC classification, stiffness and bone resorption.

Finally, limitations of the present study should be taken into consideration. Despite finding a significant relationship between the mandibular inferior cortical finding and biochemical markers of bone turnover, our study is cross-sectional. Therefore, the ability to address the issue of whether the jawbone is causally related to general bone metabolism is limited. Longitudinal studies should be undertaken to confirm a causal relationship. Furthermore, other factors affect bone turnover. We plan to investigate general bone metabolism, skeletal BMD, bone fracture, dentition status, jawbone, age, gender, nutritive intake, hormone, genetics, smoking habit, etc., synthetically.

Table 3 The result of multiple linear regression analysis using U-NTX* as a dependent variable

U-NTX (nM BCE/mM Cr)	Standardized coefficient	*p*
MIC classification ** (C1: 0, C2 or C3: 1)***	0.226	<0.01
Gender (men: 0, women: 1)	0.386	<0.01
Stiffness (%)	−0.231	<0.01
Body mass index (kg/m^2)	0.010	NS
Smoking habit (non-smoker: 0, smoker: 1)	0.100	NS
Constant		<0.01

Number of subjects=134
Coefficient of determination (R^2)=0.394 (p<0.0001)
* Urinary N-telopeptide cross-links of type I collagen
** Mandibular inferior cortex classification
*** Normal cortex (C1), mildly to moderately eroded cortex (C2), severely eroded cortex (C3)

In conclusion, our study suggests that general bone metabolism would affect the jawbone. The mandibular inferior cortical finding on dental panoramic radiographs would be useful for identifying individuals with future risk of osteoporosis.

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Conflicts of interest None.

References

1. Klemetti E, Vainio P, Lassila V et al (1993) Cortical bone mineral density in the mandible and osteoporosis status in postmenopausal women. Scand J Dent Res 101:219–223
2. Taguchi A, Tanimoto K, Suei Y et al (1996) Relationship between the mandibular and lumbar vertebral bone mineral density at different postmenopausal stages. Dentomaxillofac Radiol 25:130–135
3. Horner K, Devlin H, Alsop CW et al (1996) Mandibular bone mineral density as a predictor of skeletal osteoporosis. Br J Radiol 69:1019–1025
4. Tezal M, Wactawski-Wende J, Grossi SG et al (2000) The relationship between bone mineral density and periodontitis in postmenopausal women. J Periodontol 71:1492–1498
5. Hildebolt CF, Pilgram TK, Yokoyama-Crothers N et al (2000) Alveolar bone height and postcranial bone mineral density: negative effects of cigarette smoking and parity. J Periodontol 71:683–689
6. Wactawski-Wende J, Hausmann E, Hovey K et al (2005) The association between osteoporosis and alveolar crestal height in postmenopausal women. J Periodontol 76:2116–2124
7. Kribbs PJ (1990) Comparison of mandibular bone in normal and osteoporotic women. J Prosthet Dent 63:218–222
8. Lundstrom A, Jendle J, Stenstrom B et al (2001) Periodontal conditions in 70 year-old women with osteoporosis. Swed Dent J 25:89–96
9. Ross PD, Kress BC, Parson RE et al (2000) Serum bone alkaline phosphatase and calcaneus bone density predict fractures: a prospective study. Osteoporos Int 11:76–82
10. Garnero P, Sornay-Rendu E, Duboeuf F et al (1999) Markers of bone turnover predict postmenopausal forearm bone loss over 4 years: the OFELY study. J Bone Miner Res 14:1614–1621
11. Garnero P, Sornay-Rendu E, Claustrat B et al (2000) Biochemical markers of bone turnover, endogenous hormones and the risk of fractures in postmenopausal women: the OFELY study. J Bone Miner Res 15:1526–1536
12. Chaki O, Yoshikata I, Kikuchi R et al (2000) The predictive value of biochemical markers of bone turnover for bone mineral density in postmenopausal Japanese women. J Bone Miner Res 15:1537–1544
13. Iki M, Akiba T, Matsumoto T et al (2004) Reference database of biochemical markers of bone turnover for the Japanese female population. Japanese Population-based Osteoporosis (JPOS) Study. Osteoporos Int 15:981–991
14. Klemetti E, Kolmakov S, Kroger H (1994) Pantomography in assessment of the osteoporosis risk group. Scand J Dent Res 102:68–72
15. Taguchi A, Suei Y, Ohtsuka M et al (1996) Usefulness of panoramic radiography in the diagnosis of postmenopausal osteoporosis in women. Width and morphology of inferior cortex of the mandible. Dentomaxillofac Radiol 25:263–267
16. Nakamoto T, Taguchi A, Ohtsuka M et al (2003) Dental panoramic radiograph as a tool to detect postmenopausal women with low bone mineral density: untrained general dental practitioners' diagnostic performance. Osteoporos Int 14:659–664
17. Halling A, Persson GR, Berglund J et al (2005) Comparison between the Klemetti index and heel DXA BMD measurements in the diagnosis of reduced skeletal bone mineral density in the elderly. Osteoporos Int 16:999–1003
18. Taguchi A, Tsuda M, Ohtsuka M et al (2006) Use of dental panoramic radiographs in identifying younger postmenopausal women with osteoporosis. Osteoporos Int 17:387–394
19. Taguchi A, Tanimoto K, Suei Y (1993) The estimation of the radiomorphometric indices of the mandible using panoramic radiography. Dent Radiol 33:309–316 (in Japanese)
20. Genant HK, Wu CY, van Kuijk C et al (1993) Vertebral fracture assessment using a semiquantitative technique. J Bone Miner Res 8:1137–1148
21. Hans D, Hartl F, Krieg MA (2003) Device-specific weighted T-score for two quantitative ultrasounds: operational propositions for the management of osteoporosis for 65 years and older women in Switzerland. Osteoporos Int 14:251–258
22. Krieg MA, Cornuz J, Ruffieux C et al (2006) Prediction of hip fracture risk by quantitative ultrasound in more than 7000 Swiss women > or =70 years of age: comparison of three technologically different bone ultrasound devices in the SEMOF study. J Bone Miner Res 21:1457–1463
23. Tateiwa Y, Nakatsuchi Y, Tsuchigane A et al (2002) Discriminatory ability of quantitative ultrasound parameters and bone mineral density in elderly Japanese women with hip fracture. Osteoporos Int 13(Suppl 2):S2
24. Klemetti E, Kolmakov S (1997) Morphology of the mandibular cortex on panoramic radiographs as an indicator of bone quality. Dentomaxillofac Radiol 26:22–25
25. Japan Osteoporosis Society (2004) Osteoporosis Jpn 12:191–207 (in Japanese)
26. Stein GS, Lian JB, Qwen TA (1990) Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB J 4:3111–3123
27. Hanson DA, Weis MA, Bollen A-M et al (1992) A specific immunoassay for monitoring human bone resorption: quantitation of type I collagen cross-linked N-telopeptides in urine. J Bone Miner Res 7:1251–1258
28. Taguchi A, Sanada M, Krall E et al (2003) Relationship between dental panoramic radiographic findings and biochemical markers of bone turnover. J Bone Miner Res 18:1689–1694
29. Bollen A-M, Taguchi A, Hujoel PP et al (2000) Case-Control study on self-reported osteoporotic fractures and mandibular cortical bone. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 90:518–524
30. Taguchi A, Ohtsuka M, Nakamoto T et al (2007) Identification of post-menopausal women at risk of osteoporosis by trained general dental practitioners using panoramic radiographs. Dentomaxillofac Radiol 36:149–154
31. Shimano T, Suzuki Y, Sasaki T (2002) Long-term trend of dental radiographic examination in Japan—analysis on health insurance data. Dent Radiol 42:9–21 (in Japanese)
32. American Dental Association Survey Center (2000) 2000 survey of dental practice: characteristics of dentists in private practice and their patients. Chicago, IL
33. Tugnait ACV, Hirschmann PN (2003) Radiographic equipment and techniques used in general dental practice: a survey of general dental practitioners in England and Wales. J Dent 31:197–203

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Original Article

Role of Activated Natural Killer Cells in Oral Diseases

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SUMMARY: Many of the protective immune responses of old people are impaired and this leads to an increased risk of oral bacterial infections. Little is known about the interaction between the systemic immune response on one hand and oral infections and oral diseases on the other. Here, we conducted an epidemiological study of the independent elderly to determine the relationships between activated natural killer (NK) cells and oral bacterial infections: oral diseases such as dental caries and periodontal disease. One hundred independent elderly people aged 77 years old (53 males, 47 females) were examined. Blood samples were drawn, and activated NK cells were evaluated using CD16, CD56, and CD69 monoclonal antibodies with flow cytometry. Bacterial counts for oral streptococci, lactobacillus, and opportunistic pathogens were performed using culture techniques. Oral disease examinations were performed by dentists. A larger percentage of $CD69^+$NK cells ($CD16^+CD56^+$) showed significant correlations to the isolation numbers of total streptococci ($r = 0.409$, $P < 0.01$), the species numbers of opportunistic pathogens ($r = -0.318$, $P < 0.01$), the numbers of decayed teeth ($r = -0.223$, $P < 0.05$), and the amount of bridge work ($r = 0.219$, $P < 0.05$). A higher proportion of $CD69^+$NK cells is associated with the incidence of dental caries and the number of opportunistic pathogens and total streptococci in the oral cavity of the elderly. This suggests that the proportionate number of $CD69^+$NK cells may be a useful indicator for oral infection in elderly subjects.

INTRODUCTION

The elderly population is currently rapidly increasing in industrialized countries. In Japan, the proportion of the population >65 years old was 7.1% in 1970 but it is predicted to be 29.6% by 2030 (1). Accordingly, the number of bedridden elderly requiring systemic care in residential and nursing homes is also increasing. Reports show that institutionalized elderly have poorer oral health than those who live independently at home (2,3). Moreover, changes in microflora related to poor oral health include an increase in the prevalence of bacteria and may also contribute to the development of pneumonia (4), as the aspiration of bacteria present in oropharyngeal flora moves them into the respiratory tract; therefore, their presence is a risk factor for the elderly and compromised hosts. Consequently, the oral cavity is a reservoir for pathogenic bacteria where re-colonization may infect systemic organs.

Dental caries and periodontal diseases are a major problem for the elderly and are significantly associated with tooth loss (5-8). Several species of bacteria including *Streptococcus mutans*, *Streptococcus sobrinus*, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* are pathogens related to dental caries and periodontal disease in humans (9-12). However, definitive useful indicators or predictors for dental caries and periodontal disease have not been reported.

Natural killer (NK) cells are instrumental in the innate immune response for early production of gamma interferon (IFN-γ) and other cytokines necessary to control bacterial, parasitic and viral infections (13,14). Reports show that products prepared from broth extracts of Gram-positive bacteria, e.g., streptococci, staphylococci, and lactobacilli, activate human NK cells (15,16). Oral streptococci are the principal commensal bacteria that construct normal biofilms and play a role in the resistance to colonization by invading opportunistic pathogens in the oral cavity (17,18). Therefore, these Gram-positive bacteria interact with the human immune system and are competitors with the opportunistic pathogens on the mucosal tissues in the microbial ecosystem of the oral cavity. Individuals with either inherited or acquired immune deficiency are subject to increased risks for dental disease (19,20). However, it is not known if there is a relationship between the systemic immune response and oral microbial infection or oral disease.

Human blood NK cells responsible for antibody dependent cell-mediated cytotoxity constitutively express CD56 antigen and CD16. In addition, NK cells express C-type lectin receptors such as CD69 (which is an early activation marker) (21). CD69 is a type II integral membrane glycoprotein that is expressed on many activated cells of hematopoietic origin (22). The role of CD69 is to initiate cell activation. It presents a functional triggering molecule to activate NK and T cells where the cross-linking of CD69 induces cytotoxic activity and cytokine production (23). Therefore, $CD69^+$NK cells are indicators for early activated NK cells.

Here we quantitatively measured activated NK cells in the bloodstream to determine if there is a relationship between oral bacterial infection and oral commensal bacteria such as streptococci and lactobacilli and opportunistic pathogens with comparisons to $CD69^+$NK cells. We demonstrated that increased proportionate numbers of systemic activated NK cells correlate with oral disease, oral streptococci and opportunistic pathogen infections and are a possible indicator for oral infections.

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SUBJECTS AND METHODS

Elderly subjects: In 1998, a longitudinal interdisciplinary study of aging was initiated to evaluate the relationships between health status and dental status in Japan. Initially, questionnaires were sent to 4,542 residents aged 70 years old (born in 1927) in Niigata City. After the responders were divided by sex, 600 subjects were selected randomly with approximately the same numbers of each sex chosen for the baseline survey (24,25). The participants agreed to have medical and dental examinations, and they signed an informed consent form showing the protocol that was approved by the Ethics Committee of Niigata University Graduate School of Medical and Dental Science. The study was carried out according to the Declaration of Helsinki. Follow-up surveys have been carried out every year during June using the same methods as in the initial survey. Among the participants (n = 399) in the follow-up survey conducted in June 2005, 100 subjects (53 males, 47 females) were examined for the NK cell of their lymphocyte population. All subjects were 77 years old; the body height, body weight and number of teeth present were recorded, and the body mass index (BMI) was calculated for each subject. Some subjects had systemic diseases, including diabetes (9%), cerebral infarction (8%), cataract (6%), cyslipidemia (4%), osteoporosis (4%), angina pectoris (3%), leukemia (1%), hepatitis B (1%), and smoking habit (9%), but did not require special care.

Antibodies: The following monoclonal antibodies (mAb) were purchased from BD PharMingen (San Diego, Calif., USA): fluorescent isothiocyanate (FITC)-conjugated anti-human CD16 (clone 3G8) and phycoerythrin (PE)-conjugated anti-human CD69 (FN50). Phycoerythrin-cyanine 5 (PC5)-conjugated anti-human CD56 (N901), FITC, PE and PC5-conjugated mouse immunoglobulin G1 (IgG1) (clone 679.1Mc7) were purchased from Immunotech as isotype controls (Marseille, France).

Lymphocyte separation: Whole blood, 5-7 ml, was collected in sodium heparin tubes (10 ml VENOJECT; Terumo, Tokyo, Japan), diluted with an equal volume of Hanks' Balanced Salt Solution (HBSS) (Gibco Laboratories, Life Technologies; Paisley, UK), layered on a Ficoll-Conray density gradient separation solution (Lymphosepar I; Immuno-Biological Laboratories, Gunma, Japan), and centrifuged at 1,800 rpm for 30 min at room temperature. The peripheral blood mononuclear cells (PBMC) layer was removed and washed twice in HBSS. The PBMC were stained using PC5-conjugated CD56 and FITC-conjugated CD16 mAb to identify NK cells and stained with PE-conjugated CD69 mAb to detect the NK surface early activation marker (26). For each subject, PC5-, FITC- and PE-conjugated IgG1 isotype controls were used. PBMC (1×10^5) were then inoculated into v-bottomed 96-well plates (Coster, Cambridge, Mass., USA), and each was incubated with 20 μl mAb in 1% bovine serum albumin for 60 min at 4°C, washed three times with 150 μl of HBSS, fixed with 150 μl of 5% formalin/PBS solution; and stored at 4°C until flow cytometric analysis was performed. The proportions of the major subsets were determined using single and quadrant analysis. The percentage of FITC-, PC5- and PE-positive cells was measured using a FACSCalibur (Becton Dickinson, Franklin Lakes, N.J., USA) flow cytometer, and the data were analyzed using CellQuest Pro (Becton Dickinson) software.

Human saliva collection: Whole saliva samples were collected on swabs after saliva stimulation by the chewing of paraffin gum for 5 min, and the saliva was placed in transport fluid (0.4% agar, 0.15% thioglycolate/phosphate buffered saline). At the Bio Medical Laboratory (BML, Tokyo, Japan), the numbers of mutans streptococci, total streptococci, and lactobacillus microorganisms were determined.

Bacterial count: Cotton swabs containing the saliva samples from the elderly subjects were placed in transport fluid and taken to BML for analysis. Mutans streptococci, lactobacilli and total streptococci were noted to be typical cariogenic and commensal bacteria in the oral cavity. Each sample was plated onto Mitis-Salivarius agar (MS; Nippon Becton Dickinson Co. Ltd., Tokyo, Japan), modified Mitis-Salivarius agar containing 0.2 U/ml of bacitracin (MMSB) (27) or Rogosa SL agar (Nippon Becton Dickinson) using an EDDY JET spiral plating system (IUL, S.A., Barcelona, Spain) and incubated at 37°C under anaerobic conditions for 48 h. The total number (CFU) of total streptococci, mutans streptococci, and lactobacilli organisms was counted using MS, MMSB, and Rogosa SL agar. Colonies of mutans streptococci were identified by their characteristic appearance.

Tongue samples were collected from the tongue surface using a cotton swab (Seedswab No. 1) to determine if opportunistic pathogens were present. The sampling was performed by a physician from Niigata University trained in the mucosal surface sampling technique. The tongue bacterial samples were placed in transport fluid and transported to BML for analysis. Procedures were performed to detect qualitatively the following bacterial species (opportunistic pathogens): *Acinetobacter* spp., *Citrobacter freundii*, *Enterobacter agglomerans*, *Enterobacter cloacae*, *Escherichia coli*, *Haemophilus parainfluenzae*, *Klebsiella oxytoca*, *K. pneumoniae*, methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-sensitive *S. aureus* (MSSA), *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Serratia marcescens*, *Streptococcus agalactiae*, and *Stenotrophomonas maltophilia*. Each tongue sample was inoculated onto chocolate, OPA Staphylococcus and Drigalski agar plates (Nippon Becton Dickinson). The species numbers of opportunistic pathogens were counted in each sample. The plates were incubated using an atmosphere of 5% CO_2 in H_2 at 37°C for 24-48 h. Representative colonies from each plate were Gram stained, and isolates were made by the identification of characteristic appearance as well as by hemolysis, catalase and oxidase reactions (28). Isolates were suspended in 1 ml of 0.5% saline, gently shaken and tested using microbial identification kits to detect the above bacteria (ViTEK; BioMerieux Vitek Japan, Tokyo, Japan) (29).

Oral disease examinations: Dental examinations were conducted under artificial white light by four trained dentists. All subjects were examined at local community centers in Niigata City. All functioning teeth including third molars were assessed (except for partially erupted teeth). Using the World Health Organization (WHO) criteria (30), decayed teeth (DT), missing teeth (MT), and filled teeth (FT) (DMFT) scores were recorded along with findings of dental caries. The dentists assessed each patient's periodontal condition using six measurement points (mesio-buccal, buccal, disto-buccal, mesio-lingual, lingual, and disto-lingual) for each tooth. The number of sound teeth, crowns, bridges, and retained roots were counted. Intra- and inter-examiner reliability was confirmed using a kappa statistic {k = 0.56-0.92 for attachment loss (AL)}. To estimate periodontal status, rCA (the rate of sites with dental calculus), rAL4 (the rate of sites with greater than 4 mm of AL), rAL6 (the rate of sites with greater than 6

mm of AL), rPD4 {the rate of sites with greater than 4 mm of periodontal pocket (PD)}, rPD6 (the rate of sites with greater than 6 mm of PD), AL {length (mm) of attachment loss} and BOP (the rate of sites with bleeding on probing) were measured at the same six points for each tooth (31). Mouth mirrors were used incorporating a light and pressure-sensitive plastic periodontal probe set to give a constant force of 20 g and graded at 1-mm intervals (VIVACARE TPS PROBE®). In cases where a restorative margin was apical to the cement-enamel junction (CEJ), AL was measured using the observed anatomical features of the teeth; and if present, the CEJ of the adjacent tooth/teeth. The BOP was measured at six sites per tooth. Before and during the survey, examination calibrations were conducted at an institution for the aged and at the Faculty Hospital of Dentistry, Niigata University. Intra-examiner agreement ranged from 86.6 to 95.9% and from 65.8 to 94.4% for PD and AL, respectively. The kappa values ranged from 0.79 to 0.93 and from 0.56 to 0.92 for PD and AL, respectively. Thereafter, the indicators were assessed and used to estimate the periodontal status of each subject.

Statistical analysis: All encrypted data were provided by the epidemiological research group of preventive dentistry at Niigata University and analyzed by the authors. All data were analyzed using the Statistical Package for SPSS for Windows (version 100; Chicago, Ill., USA). The student's t test was used to compare the means of the parameters between males and females. Correlations between two variables were tested using the Pearson rank correlation. A P value of 0.05 or less was considered to indicate statistical significance.

RESULTS

Characteristics of elderly patients: The characteristics of elderly subjects and dental and periodontal disease status were compared between males and females. We recorded the height, weight, highest PD, highest AL, mean AL, % of sites with an AL >4 mm, and % sites with an AL >6 mm (Table 1). Males had a significantly higher AL at >6 mm than females. However, there were no other significant gender differences found in the other parameters.

Analysis of NK cells: For all patients, the proportionate number of NK ($CD56^+CD16^+$) cells in lymphocytes was 23.6 ± 13.9% and the proportion of $CD69^+$ cells in the NK cell population was 31.2 ± 14.3%. There was a significant gender difference in the proportion of NK cells in lymphocytes (male 26.9 ± 13.1%, female 19.8 ± 13.9; $P < 0.05$). There was no significant gender difference in the proportion of $CD69^+$NK cells (male 30.7 ± 14.2%, female 31.8 ± 14.7). Even though some subjects had systemic diseases and a smoking habit, there were no significant differences in the proportion of NK and $CD69^+$NK cells shown in total systemic diseases, diabetes and cerebral infarction, and smoking habit (data not shown).

Correlation of oral bacteria numbers to activated NK cells: To analyze the relationships between activated NK cells and the number of oral streptococci and lactobacilli, the bacterial numbers were measured in the saliva and compared with the proportionate numbers of NK or $CD69^+$NK cells in the peripheral blood. In addition, infection by opportunistic pathogens on the tongue was also compared with the proportion of NK or $CD69^+$NK cells. A relatively increased proportion of $CD69^+$NK cells showed a positive correlation with the isolation numbers of total streptococci ($r = 0.409$, $P < 0.01$) and a statistically negative correlation with the species numbers of opportunistic pathogens ($r = -0.318$, $P < 0.01$) (Table 2). An increased proportion of $CD69^+$NK cells showed, in males, a positive correlation with the isolation numbers of total streptococci ($r = 0.408$, $P < 0.01$) and a negative correlation with the species numbers of opportunistic pathogens ($r = -0.330$, $P < 0.05$), and in females, a positive correlation with the isolation numbers of total streptococci ($r = 0.389$, $P < 0.05$). There were no significant correlations between NK cells and any bacterial level nor between $CD69^+$NK cells and lactobacilli or mutans streptococci for either male or

Table 1. Characteristic of the subjects

	Total	Male	Female	P-value
Height (cm)	155.8 ± 7.5	161.3 ± 5.1	149.6 ± 4.2	<0.001**
Weight (kg)	54.9 ± 9.8	58.6 ± 9.9	50.8 ± 8.0	<0.001**
BMI (kg/m^2)	22.5 ± 3.2	22.4 ± 3.1	22.6 ± 3.3	0.762
Dental condition				
Number of present teeth	15.9 ± 9.8	16.3 ± 9.8	15.4 ± 9.9	0.629
Sound teeth	7.4 ± 6.8	8.6 ± 7.0	6.0 ± 6.6	0.062
Decayed teeth	0.1 ± 0.6	0.2 ± 0.8	0.1 ± 0.2	0.263
Filled teeth	8.4 ± 5.5	7.6 ± 4.8	9.3 ± 6.2	0.127
Missing teeth	12.4 ± 9.3	12.1 ± 9.3	12.8 ± 9.5	0.698
Highest PD	5.5 ± 1.9	6.0 ± 1.8	5.0 ± 1.8	0.011*
Mean PD	2.3 ± 0.6	2.4 ± 0.6	2.2 ± 0.5	0.068
% site with PD ≧4 mm	12.8 ± 13.7	14.9 ± 13.6	10.3 ± 13.5	0.109
% site with PD ≧6 mm	2.5 ± 5.1	3.3 ± 6.0	1.5 ± 3.6	0.084
Hightest AL	7.3 ± 2.3	8.0 ± 2.3	6.4 ± 2.0	0.001**
Mean AL	3.6 ± 1.2	3.9 ± 1.2	3.3 ± 1.0	0.021*
% site with AL ≧4 mm	43.6 ± 30.8	49.5 ± 29.4	36.7 ± 31.2	0.049*
% site with AL ≧6 mm	12.0 ± 19.1	15.8 ± 21.3	7.5 ± 15.2	0.040*
% site with BOP	11.3 ± 13.4	11.1 ± 13.0	11.5 ± 14.1	0.893

P values evaluate gender differences.
*$P < 0.05$.
**$P < 0.01$.
BMI, body mass index; PD, probing depth; AL, attachment level; BOP, bleeding on probing.

Table 2. Correlation between oral bacterial numbers and NK cell

Oral bacteria status	Total		Male		Female	
	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$
Opportunistic pathogens	0.167 (0.112)	−0.318 (0.002)**	0.219 (0.123)	−0.330 (0.018)*	0.088 (0.586)	−0.307 (0.051)
Total streptococci	0.139 (0.190)	0.409 (0.0001)**	−0.028 (0.847)	0.408 (0.004)**	−0.195 (0.216)	0.389 (0.011)*
Lactobacilli	0.143 (0.176)	0.116 (0.272)	0.202 (0.164)	−0.044 (0.763)	0.121 (0.446)	−0.230 (0.142)
Mutans streptococci	0.072 (0.497)	0.113 (0.209)	0.150 (0.303)	0.193 (0.183)	0.065 (0.681)	−0.006 (0.968)

$CD56^+CD16^+$, NK cell; $CD69^+/CD56^+CD16^+$, activated NK cell.
Values are Pearson correlation coefficient.
*$P < 0.05$.
**$P < 0.01$.

Table 3. Correlation between tooth/restoration status and NK cell

Coronal status	Total		Male		Female	
	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$
Sound teeth	0.119 (0.238)	−0.102 (0.310)	0.010 (0.946)	−0.071 (0.614)	0.151 (0.312)	−0.129 (0.387)
Crown	−0.045 (0.654)	0.152 (0.132)	0.004 (0.978)	0.362 (0.008)**	−0.036 (0.812)	0.004 (0.977)
Bridge (abutment)	0.013 (0.897)	0.219 (0.028)*	0.053 (0.707)	0.169 (0.227)	0.044 (0.769)	0.259 (0.079)
Retained roots	0.123 (0.224)	−0.123 (0.223)	0.190 (0.173)	−0.123 (0.381)	0.039 (0.795)	−0.124 (0.407)
Decay teeth	−0.015 (0.880)	−0.223 (0.026)*	−0.043 (0.757)	−0.286 (0.038)*	−0.076 (0.613)	−0.119 (0.425)
Missing teeth	−0.063 (0.532)	0.001 (0.995)	−0.018 (0.900)	−0.037 (0.794)	−0.094 (0.529)	0.038 (0.802)
Filling teeth	−0.023 (0.824)	0.175 (0.082)	0.020 (0.887)	0.267 (0.053)	0.017 (0.912)	0.091 (0.543)

$CD56^+CD16^+$, NK cell; $CD69^+/CD56^+CD16^+$, activated NK cell.
Values are Pearson correlation coefficient.
*$P < 0.05$.
**$P < 0.01$.

Table 4. Correlation between clinical periodontal measurement and NK cell

Periodontal status	Total		Male		Female	
	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$	$CD56^+CD16^+$	$CD69^+/CD56^+CD16^+$
Number of teeth present	0.069 (0.498)	0.015 (0.885)	0.013 (0.925)	0.059 (0.675)	0.105 (0.482)	−0.029 (0.846)
Hightest PD	−0.002 (0.983)	0.085 (0.430)	−0.198 (0.178)	0.265 (0.069)	0.061 (0.703)	−0.066 (0.681)
Mean PD	0.022 (0.837)	0.028 (0.796)	−0.042 (0.777)	0.054 (0.717)	−0.010 (0.953)	0.031 (0.846)
% site with PD ≧ 4 mm	−0.014 (0.893)	0.040 (0.708)	−0.085 (0.567)	0.075 (0.615)	−0.032 (0.843)	0.032 (0.844)
% site with PD ≧ 6 mm	0.036 (0.735)	−0.020 (0.855)	−0.046 (0.756)	0.067 (0.649)	0.061 (0.706)	−0.146 (0.362)
Hightest AL	0.016 (0.878)	0.011 (0.919)	−0.066 (0.657)	0.100 (0.498)	−0.080 (0.620)	−0.042 (0.794)
Mean AL	0.078 (0.466)	−0.087 (0.419)	0.086 (0.560)	−0.100 (0.498)	−0.065 (0.684)	−0.031 (0.846)
% site with AL ≧ 4 mm	0.041 (0.704)	−0.023 (0.828)	0.077 (0.603)	−0.032 (0.828)	−0.100 (0.534)	0.019 (0.908)
% site with AL ≧ 6 mm	0.078 (0.468)	−0.144 (0.179)	0.050 (0.734)	−0.162 (0.272)	−0.010 (0.951)	−0.088 (0.585)
% site with BOP	0.061 (0.568)	0.016 (0.880)	0.097 (0.511)	0.127 (0.390)	0.038 (0.815)	−0.100 (0.534)

$CD56^+CD16^+$, NK cell; $CD69^+/CD56^+CD16^+$, activated NK cell.
Values are Pearson correlation coefficient.
PD, probing depth; AL, attachment level; BOP, bleeding on probing.

female subjects.

Correlation of dental disease status to activated NK cells: To analyze the relationships between activated NK cells and oral disease status, decayed teeth and teeth cured by bridges and crowns were measured and compared with the proportion of $CD69^+$NK cells. A relatively increased proportion of $CD69^+$NK cells showed significant correlations with bridges ($r = 0.219$, $P < 0.05$) and decayed teeth ($r = -0.223$, $P < 0.05$) (Table 3). An increased $CD69^+$NK cell population showed significant correlations with crowns ($r = 0.362$, $P < 0.01$) and decayed teeth ($r = -0.286$, $P < 0.05$) in male subjects (Table 3), while, increased populations in females showed a correlation with bridges ($r = 0.259$) but was not significant in other oral disease status observations (Table 3). There were no other significant correlations between parameters of periodontal diseases and NK cell or $CD69^-$NK cell proportions in male and female subjects (Table 4). The numbers of subjects having partial dentures and full dentures were 33 and 17, respectively. There was no significant difference between subjects with partial or full dentures and subjects without in terms of the proportions of NK cells and $CD69^+$NK cells.

DISCUSSION

The oral carriage of bacteria causing pneumonia such as *K. pneumoniae*, *Pseudomonas* sp. and *Staphylococcus* sp. is low in healthy subjects and higher in immunodeficient, myelosuppressed and elderly subjects requiring care (7,32-34). Smith et al. report that coagulase-negative staphylococcus spp. emerged in many debilitated elderly patients and in those with oral Crohn's disease (35). *Candida* spp. levels were also higher in the oral cavities of critically ill patients than in women who were considered to be healthy (36). It is possible that individuals who have opportunistic pathogens may suffer a deterioration in host defenses. Thus, we speculate that

the change in systemic conditions involving immune activity may interact with and restrict oral microbial ecology. In this study, we found the activated NK cells to be a responsible immune indicator for the incidence of oral disease and oral bacterial infection in elderly subjects.

Products prepared from Gram-positive bacteria including streptococci, staphylococci, and lactobacilli activate human NK cells (15,16) and induce CD14-independent pathways to stimulate human monocytes (23,37). Toll-like receptor 2 is implicated in the recognition of Gram-positive bacterium-derived lipoteichoic acid (LTA) and peptidoglycan (PG) (38). The proportionate increase of $CD69^+$NK cells correlates with infection by Gram-positive bacteria such as oral streptococci and lactobacilli. However, we show a correlation to the number of total streptococci but not to the numbers of lactobacilli and mutans streptococci in the saliva. Moreover, the proportion of $CD69^+$NK cells did not correlate with periodontal disease status. This suggests that systemic activated NK cells are susceptible to oral total streptococci, the primary organisms in the oral cavity, but not to lactobacilli, mutans streptococci or the pathogens that cause periodontal disease in the elderly.

The correlation between activated NK cells and microbial infection and dental caries was greater in males than females. The reason for this is not understood. However, Willemsen et al. reported that mental and cold stress increased NK cells and secretory immunoglobulin A (sIgA) in males but not in females (39). This may partially explain the gender differences in the correlation with activated NK cells.

We show that lower proportionate numbers of $CD69^+$NK cells suggest that the oral streptococcal level may decrease, the opportunistic pathogen level may increase, and dental caries and pneumonia may develop in the future. Therefore, our data suggest that the proportion of active $CD69^+$NK cells may be a useful indicator of oral infection in elderly subjects. However, it is not clear how $CD69^+$NK cells are associated with the number of total streptococci and with dental status. Further studies are required to clarify these mechanisms. A limitation of our study is the bias introduced by the level of participation. The participants were 100 subjects (25.1%) out of a target population of 399 subjects; therefore, the data may be somewhat biased, as the present elderly subjects were generally in good health and might have been more eager and/or able to participate in this survey. Thus, our findings may indicate an association in generally healthy elderly subjects. Therefore, additional follow-up studies with these elderly subjects are required to provide clear findings of interest.

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REFERENCES

1. National Institute of Population and Social Security Research (2002): Population Projections for Japan: 2001-2050. Health and Welfare Statistics Association, Tokyo.
2. Steele, J.G., Sheiham, A., Marcenes, W., et al. (1998): National diet and nutrition survey: people aged 65 years and over. vol. 2. Report of the Oral Health Survey. Stationery Office, London.
3. Simons, D., Kidd, E.A. and Beighton, D. (1999): Oral health of elderly occupants in residential homes. Lancet, 353, 1761.
4. Yoneyama, T., Yoshida, M., Matsui, T., et al. (1999): Oral care and pneumonia. Oral Care Working Group. Lancet, 354, 515.
5. Richards, W., Ameen, J., Coll, A.M., et al. (2005): Reasons for tooth extraction in four general dental practices in South Wales. Br. Dent. J., 198, 275-278.
6. Fure, S. (2003): Ten-year incidence of tooth loss and dental caries in elderly Swedish individuals. Caries Res., 37, 462-469.
7. Ong, G. (1998): Periodontal disease and tooth loss. Int. Dent. J., 48, 233-238.
8. Oliver, R.C. and Brown, L.J. (1993): Periodontal diseases and tooth loss. Periodontol. 2000, 2, 117-127.
9. Köhler, B. and Persson, M. (1991): Salivary levels of mutans streptococci and lactobacilli in denture 80- and 85-year-old Swedish men and women. Community Dent. Oral Epidemiol., 19, 352-356.
10. Loesche, W.J., Schork, A., Terpenning, M.S., et al. (1995): Factors which influence levels of selected organisms in saliva of older individuals. J. Clin. Microbiol., 33, 2550-2557.
11. Socransky, S.S. and Haffajee, A.D. (1992): The bacterial etiology of destructive periodontal disease: current concepts. J. Periodontol., 63, 322-331.
12. Kolenbrander, P.E. and London, J. (1993): Adhere today, here tomorrow: oral bacterial adherence. J. Bacteriol., 175, 3247-3252.
13. Biron, C.A., Nguyen, K.B., Pien, G.C., et al. (1999): Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu. Rev. Immunol., 17, 189-220.
14. Cooper, M.A., Fehniger, T.A., Turner, S.C., et al. (2001): Human natural killer cells: a unique innate immunoregulatory role for the $CD56^{bright}$ subset. Blood, 97, 3146-3151.
15. Lapham, C., John, P.A. and Tomar, R.H. (1986): The mechanism of enhancement of natural killer cell activity by soluble streptococcal products. Clin. Immunol. Immunopathol., 40, 335-346.
16. Haller, D., Serrant, P. and Granato, D. (2002): Activation of human NK cells by staphylococci and lactobacilli requires cell contact-dependent costimulation by autologous monocytes. Clin. Diagn. Lab. Immunol., 9, 649-657.
17. Bryskier, A. (2002): Viridans group streptococci: a reservoir of resistant bacteria in oral cavities. Clin. Microbiol. Infect., 8, 65-69.
18. Tada, A., Senpuku, H., Motozawa, Y., et al. (2006): Association between commensal bacteria and opportunistic pathogens in the dental plaque of elderly individuals. Clin. Microbiol. Infect., 12, 776-781.
19. Madigan, A., Murray, P.A., Houpt, M., et al. (1996): Caries experience and cariogenic markers in HIV-positive children and their siblings. Pediatr. Dent., 18, 129-136.
20. Tomar, S.L., Swango, P.A., Kleinman, D.V., et al. (1995): Loss of periodontal attachment in HIV-seropositive military personnel. J. periodontol., 66, 421-428.
21. Testi, R., D'Ambrosio, D., Maria, R., et al. (1994): The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol. Today, 15, 479-483.
22. Marzio, R., Mauël, J. and Betz-Corradin, S. (1999): CD69 and regulation of the immune function. Immunopharmacol. Immunotoxicol., 21, 565-582.
23. Cauwels, A., Wan, E., Leismann, M., et al. (1997): Coexistence of CD14-dependent and independent pathways for stimulation of human monocytes by gram-positive bacteria. Infect. Immun., 65, 3255-3260.
24. Yamaga, T., Yoshihara, A., Ando, Y., et al. (2002): Relationship between dental occlusion and physical fitness in an elderly population. J. Gerontol. A Biol. Sci. Med. Sci., 57, M616-M620.
25. Yoshihara, A., Hanada, N. and Miyazaki, H. (2003): Association between serum albumin and root caries in community-dwelling older adults. J. Dent. Res., 82, 218-222.
26. Yokoyama, W.M. (1999): Natural killer cells. p. 575. *In* Paul W.E. (ed.), Fundamental Immunology. Lappincent-Reven, New York.
27. Tsuha, Y., Hanada, N., Asano, T., et al. (2004): Role of peptide antigen for induction of inhibitory antibodies to *Streptococcus mutans* in human oral cavity. Clin. Exp. Immunol., 137, 393-401.
28. Murray, R.G.E., Brenner, D.J., Bryant, M.R., et al. (1989): Bergey's Manual of Systematic Bacteriology. vol. 1-4. Lippincott, Williams & Wilkins, Philadelphia.
29. Salam, M.A., Senpuku, H. and Nomura, Y. (2001): Isolation of opportunistic pathogens in dental plaque, saliva and tonsil samples from elderly. Jpn. J. Infect. Dis., 54, 193-195.
30. World Health Organization (1997): Oral Health Surveys Basic Methods. 4th ed. World Health Organization, Geneva.
31. Ogawa, H., Yoshihara, A., Hirotomi, T., et al. (2002): Risk factors for periodontal disease progression among elderly people. J. Clin. Periodontol., 29, 592-597.
32. Minah, G.E., Rednor, J.L., Peterson, D.E., et al. (1986): Oral succession of gram-negative bacilli in myelosuppressed cancer patients. J. Clin. Microbiol., 24, 210-213.
33. Bergmann, O.J., Kilian, M. and Ellegaard, J. (1989): Potentially pathogenic microorganisms in the oral cavity during febrile episodes in

immunocompromised patients with haematologic malignancies. Scand. J. Infect. Dis., 21, 43-51.
34. Senpuku, H., Sogame, A., Inoshita, E., et al. (2003): Systemic diseases in association with microbial species in oral biofilm from elderly requiring care. Gerontology, 49, 301-309.
35. Smith, A.J., Jackson, M.S. and Bagg, J. (2001): The ecology of *Staphylococcus* species in the oral cavity. J. Med. Microbiol., 50, 940-946.
36. Mitchell, K.G., Bradley, J.A. and Ledingham, I.M. (1982): Candida colonization of the oral cavity. Surg. Gynecol. Obstet., 154, 870-874.
37. Cuzzola, M., Mancuso, G., Beninati, C., et al. (2000): Beta 2 integrins are involved in cytokine responses to whole Gram-positive bacteria. J. Immunol., 164, 5871-5876.
38. Michelsen, K.S., Aicher, A. and Mohaupi, M. (2001): The role of Toll-like receptors (TLRs) in bacteria-induced maturation of murine dendritic cells (DCs). Peptidoglycan and lipoteichoic acid are inducers of DC maturation and require TLR2. J. Biol. Chem., 276, 25680-25686.
39. Willemsen, G., Carroll, D., Ring, C., et al. (2002): Cellular and mucosal immune reactions to mental and cold stress: associations with gender and cardiovascular reactivity. Psychophysiology, 39, 222-228.

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Original Article

Impact of routine oral care on opportunistic pathogens in the institutionalized elderly

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Routine oral hygiene is important for the control of opportunistic pathogens in the oral cavity of institutionalized elderly individuals. We evaluated the effects of routine oral care on opportunistic pathogens at various time points after admission to a nursing home. Twenty-five elderly subjects living in the nursing home (mean age: 86.0±10.4 years) participated in the study. Caregivers and dental hygienists cleaned the teeth, dentures, tongue, and mucosa after each meal using both routine and professional oral care techniques. Opportunistic pathogens were collected from the teeth, tongue, and mucosal surfaces using a cotton swab; and the species of microbes were determined and the numbers were counted following cultivation on selective agar. Regular oral care including professional oral care was found to be effective for reducing infections by many kinds of opportunistic pathogens on the teeth surfaces and the oral environment without food residue during a long-term study (6 months). Further, this care after 1 month significantly reduced infections by opportunistic pathogens on mucosal surfaces in subjects without dentures; however, this was not observed in those with dentures. Our data shows the importance of regular oral care in cleaning hard and soft surfaces of the oral cavity improves the oral health of the institutionalized elderly.

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Introduction

In Japan, the number of elderly people is steadily increasing where those over the age of 65 will account for approximately 25% of the population by 2025[1]. Accordingly, the number of bedridden elderly requiring systemic care in residential and nursing homes will also increase. Reports show institutionalized elderly individuals have poorer oral health than those who live independently at home[1-3]. Further, the oral cavity is thought to be a potential reservoir of opportunistic pathogens that are risk factors for pneumonia in the elderly[4-6]. Further, studies show a higher prevalence of nosocomial and Gram-negative enteric bacilli pathogens in institutionalized elderly patients with severe pneumonia[7,8]. El-Solh *et al.* reported respiratory pathogens colonizing dental plague were implicated in the infections of the lower respiratory tract in institutionalized elderly subjects[9].

There are possible links with poor hygiene and host-defense problems to an increased incidence of pneumonia in institutionalized elderly patients[10-12]. Therefore, oral hygiene is considered to be important to control opportunistic pathogens on teeth and mucosal surfaces; and some studies indicate oral hygiene for hospitalized elderly patients reduces the risk of nosocomial pneumonia[3,13,14]. Thus, regular dental care may be effective in reducing the numbers of dental and respiratory bacteria for the elderly residents in long-term care facilities. Although the effects of oral care

have been reported, few studies have surveyed the different opportunistic pathogens in institutionalized elderly subjects between, before and after receiving regular dental care provided by care-givers and dental hygienists. In this study, we isolated opportunistic pathogens from the teeth, tongue, and mucosal surfaces from elderly subjects requiring systemic care at various time points before and after regular and professional oral care. The purpose of this study was to evaluate the effects of oral care on opportunistic pathogen populations in institutional elderly people.

Materials and Methods

Subjects

Beginning 2 weeks after entering a Toshima-ward, Tokyo nursing home, 25 residents (mean age: 86.0± 10.4 years; 6 males, 19 females) who required long-term nursing care participated in this study. This nursing home was new (established May, 2004) with a capacity of 62. The study was conducted from October 2004 to May 2005 where the subjects were randomly selected from the residents of the same floor using a random-numbers table and held blind from the investigators. The subjects were in two groups; those requiring little care, *i.e.* not bedridden or confined to bed (n = 8); and those requiring intensive care, *i.e.* confined to their bed (n = 17). Prior consent was obtained from all subjects. The study was approved by the Ethics Committee of the Tokyo Medical and Dental University and performed according to the rules of the Helsinki Declaration. Dental examinations to determine the presence of dental caries, periodontal pocket depths, dental calculus, remaining food residues, and other typical oral conditions were performed using artificial white light by trained dentists before the study and during routine professional oral care. Four dentists assessed the subject's dental and periodontal condition using six measurements points for each tooth: mesiobuccal, buccal, distobuccal mesiolingual, lingual, and distolingual.

Oral care

At the initial examination, all patients had a routine dental and medical examination. Oral care techniques by dental hygienists and care givers were standardized before beginning oral care. For daily oral care, subjects who were able used the sink facilities in their rooms and performed standard oral hygiene three times a day by themselves; and cleaning status was confirmed by the care givers examining the oral cavity. Whereas the other subjects were performed by care givers three times a day and assisted in oral cleaning with tooth brushing, brushing of denture surfaces and oral rinsing with tap water. The patients cleaned their teeth, dentures, tongue, and mucosa surfaces after each meal using routine oral care techniques. Further, for 20 minutes twice per month, dental hygienists provided professional care such as removing oral calculus with a scaler, dental brushing of teeth surfaces, mucosal cleaning with a sponge brush, denture cleaning, and oral washing with 0.5% povidone-iodine solution (Isodine-Gargle, Meiji seika, Tokyo, Japan) in addition to the daily oral care. The routine oral care including professional care was performed for 6 months. Daily oral care without professional care was performed from entering the institution to the first sampling. No antibiotic therapy was administered during the 2 weeks before the start of this study and during the 6 month study period; and none of the subjects suffered from severe infections or systemic diseases. There was no information about antibiotic therapy for the subjects before entering the institution. The percentage of subjects who retained their own teeth was 36% (9/25). None of the subjects dropped-out of the study.

Bacterial sampling

Supragingival plaque samples were collected from the posteroanterior buccal surface of the upper right second premolar, the buccal surfaces of the upper right second premolar and first molar using a cotton swab (Seedswab No. 1, Eiken Chemical Co., Ltd., Tokyo); and transferred to 1 ml of reduced transport fluid (0.4% agar, 0.15% thioglycollate/phosphate-buffered saline) in sterile bottles. For edentulous subjects who used complete dentures (n = 12), the samples were collected from the same regions of the upper right second premolar and first molar of the complete dentures. For edentulous subjects not using dentures, plaque samples were not collected. Subjects using partial denture (n = 2) and not having any of the above mentioned teeth were sampled from the opposite side or other remaining teeth. Samples were also collected by swabbing five times from the center of the tongue and right buccal surface of the oral mucosa. All samples were taken before professional care by a dental hygienist. After placement in transport fluid, the samples were immediately transported to the Biomedical Laboratory (BML, Tokyo, Japan) for analysis to detect opportunistic pathogenic bacteria.

Identification of bacteria and fungi

The isolated bacteria and fungi from the plaque, the tongue, and mucosal surfaces were identified using culture procedures[15]. The samples were pour plated on chocolate agar, blood agar, OPA staphylococcus, and Drigalski agar plates (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan); and were incubated in an atmosphere of 5% CO_2 at 37°C for 24-48 hours. Representative colonies from each plate were isolated and analyzed using Gram stain, hemolysis, and oxidase reactions[16]. The colonies were suspended in 1 ml 0.5% saline; gently shaken; and tested using microbial identification kits (VITEK; BioMerieux Vitek Japan, Tokyo, Japan)[16]. The bacteria and fungi detected in the samples are shown in Table 1.

Statistical procedures

All data were analyzed using the Statistical Package for Social Science (SPSS) version 11.5. The proportion of elderly subjects in the two groups was compared using the Chi-square and Wilcoxon signed-rank tests for equal and unequal variations. A *p*-value of less than 0.05 was considered to be significant.

Results

Samples were not taken from the teeth, tongue and mucosal surfaces of two elderly subjects respectively at 4 and 6 months after the beginning of oral care owing to poor health. One month after starting oral care, the numbers of opportunistic pathogens on the teeth, tongue, and oral mucosal surfaces decreased in 6 of 21 (28.6%), 11 of 25 (44.0%), and 10 of 25 (40.0%) of the subjects, respectively, in comparison with their numbers at the beginning of the study (Fig.1). The proportion of subjects with decreasing opportunistic pathogens on teeth surfaces was significant at 4 (12/19, 63.2%, p = 0.027) and 6 (13/19, 68.4%, p = 0.011) months as compared to after 1 month (Fig. 1). In contrast, the proportion of subjects with decreased numbers (13/23, 56.5%) on the mucosa surfaces after 4 months were greater but were not significantly higher than at 1 month (10/25, 40.0%) after the beginning of oral care (Fig.1). The proportion of subjects with decreasing numbers on the tongue after 4 months (6/23, 26.1%) was fewer and those after 6 months (13/23, 56.5%) increased as compared to those after 1 month (11/25, 44.0%); but none were significantly different. *Candida albicans* tended to remain on all surface areas at varying sampling times after professional care but other opportunistic pathogens did not.

The infection or accumulation of multiple species of opportunistic pathogens is a risk factor for respiratory tract infections in institutionalized elderly subjects. Therefore, detection of opportunistic pathogens was performed using a qualitative analysis to isolate multiple species. The proportion of elderly subjects where more than four species and strains of opportunistic pathogens were isolated was 10/21 (47.6%), 11/25 (44.0%) or 11/25 (44.0%) on the teeth, tongue and mucosal surfaces, respectively, before the beginning of professional care. To evaluate the effects of oral care in the elderly subjects, the proportion of subjects with

Table 1. Lists of species and strains of opportunistic pathogens detected on tooth, tongue and oral mucosa

Acinetobacter calcoaceticus	Acinetobacter sp.
Bacillus cereus	Chryseobacterium sp.
Candida albicans	Corynebacterium sp.
Candida glabrata	Enterobacter sp.
Candida tropicalis	Enterococcus sp.
Chryseobacterium indologenes	Pseudomonas sp.
Citrobacter freundii	
Citrobacter koseri	β-Streptococcus
Enterobacter cloacae	
Escherichia coli	
Haemophilus influenzae	
Klebsiella pneumoniae	
MSSA (methicillin-sensitive *S. aureus*)	
Pseudomonas aeruginosa	
Serratia marcescens	
Stenotrophomonas maltophilia	
Xanthomonas maltophilia	

The strains and species of opportunistic pathogens were counted in each sample.

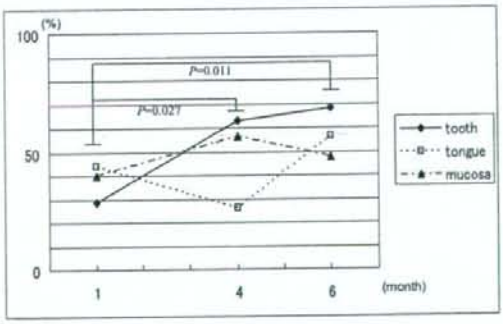


Fig. 1. The effects of routine oral care on the numbers of opportunistic pathogens compared to the numbers on initial examination. The number of subjects with decreasing numbers of opportunistic pathogens on teeth (n = 21, 19, and 19), tongue (n = 25, 23, and 23), and oral mucosa (n = 25, 23, and 23) surfaceswith 1, 4, and 6 months of treatment respectively are shown. Numbers of opportunistic pathogens were 3.2±1.5, 3.6±1.0 and 3.3±1.2 for teeth, tongue, and oral mucosal surfaces, respectively, compared to prior to dental care. Asterisks denote significant difference in the chi-square test (p < 0.05, 1 month versus 4 or 6 months for each sample).

more than four species and strains of opportunistic pathogens was employed as an indicator. On teeth surfaces the numbers significantly decreased after 4 (2/19; 10.5%. $p = 0.012$) and 6 months (1/19; 5.3%, $p = 0.003$) in comparison to before the beginning of professional care (Fig. 2). On mucosal surfaces the opportunistic pathogens decreased after 4 and 6 months (3/23; 13.0% and 4/23; 17.4%) but not significantly. And there were no significant differences for the tongue surface. The comparison was confirmed using the mean±SD of opportunistic pathogens numbers as analyzed using the Wilcoxon signed-rank test (see below) on the teeth surfaces. The opportunistic pathogen numbers (2.4±0.8) at 6 months after professional oral care was significantly lower than before the start of professional oral care (3.2±1.5) on the teeth surfaces ($p = 0.028$). However, there were no significant differences in the other comparisons. Therefore, long-term professional oral care is significant to effectively decrease opportunistic pathogen numbers on teeth surfaces in comparison to short-term professional oral care.

The proportion of elderly subjects having decreased numbers of opportunistic pathogens on mucosal surfaces was significantly lower in patients with dentures (2/13, 15.4%) than in those without dentures (8/12, 66.7%) at 1 month ($p = 0.013$) but not at 4 and 6 months after the beginning of oral care (Fig. 3C). However, there were no significant differences for the teeth and tongue surfaces between subjects with and without dentures at various time points (Fig. 3 A and B). The number of subjects with decreasing num-

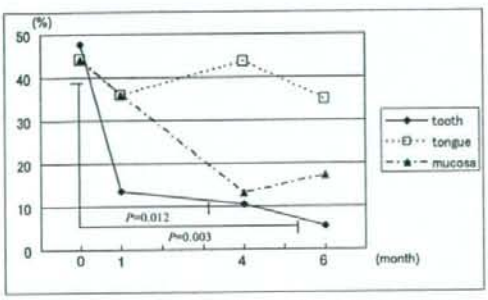


Fig. 2. The effects of routine oral care on the reduction of more than four types of opportunistic pathogens.
The number of subjects with more than four species and strains of opportunistic pathogens detected on the teeth, tongue, and oral mucosal surfaces at 0, 1, 4, and 6 months are shown. Asterisks denote significant difference in the chi-square test ($p < 0.05$, 0 month versus 1, 4 or 6 months for each sample).

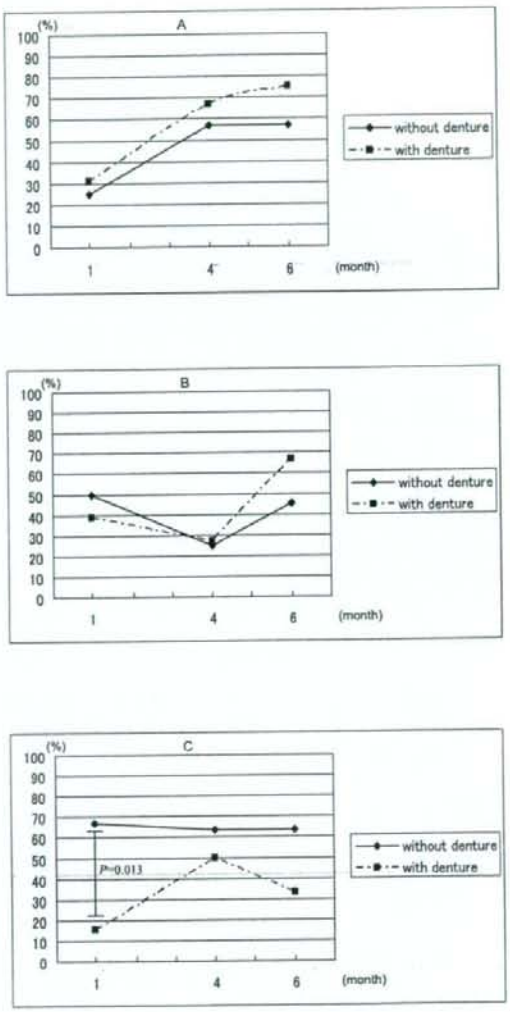
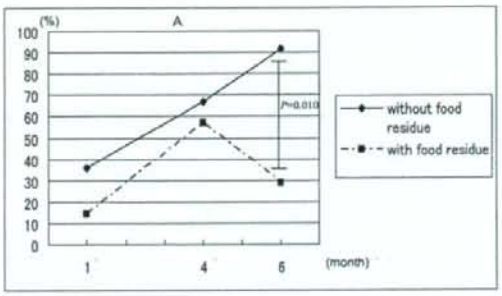
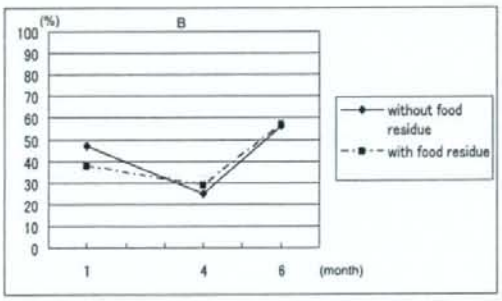


Fig. 3. The effects of routine oral care in subjects with and without dentures.
The number of subjects with decreasing numbers of opportunistic pathogens on the teeth (A), tongue (B), and mucosal (C) surfaces with and without dentures at 1, 4, and 6 months are shown. The number of subjects with dentures were 13, 12 and 12 for teeth, 13, 11 and 12 for the tongue, and 13, 12 and 12 for mucosa at 1, 4 and 6 months, respectively, after beginning professional care. The numbers of opportunistic pathogens were 2.7±1.5 and 3.8±1.3 for teeth, 3.7±0.9 and 3.5±1.3 for the tongue, and 3.1±0.9 and 3.4±1.1 on oral mucosal surfaces of elderly subjects with and without dentures, respectively, before the beginning of the study. Asterisks denote significant difference in the chi-square test ($p < 0.05$, with dentures vs. without dentures at 1, 4 or 6 months for each sample).

bers of opportunistic pathogens in elderly subjects with food residue (2/7, 28.6%) was significantly lower than those in subjects without food residue (11/12, 91.7%) on the teeth surfaces at 6 months ($p = 0.010$) (Fig. 4A). However, there were no significant differences among the patients with and without food residues on the tongue and mucosa at each sampling period after the beginning of oral care (Fig. 4B, C). Moreover, there were no significant differences between the two groups (non- bedridden and bedridden) in all data (data not shown) and among other dental and periodontal parameters.





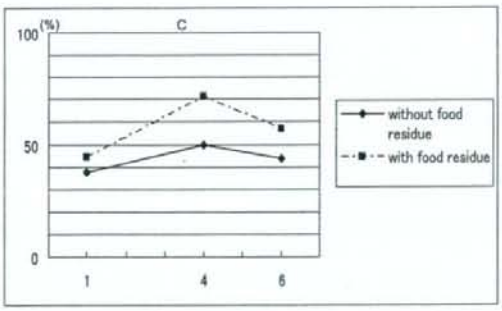


Fig. 4. The effects of routine oral care for subjects with and without food residue.
The proportions of subjects with decreasing numbers of opportunistic pathogens on the teeth (A), tongue (B), and mucosal (C) surfaces with and without food residues at 1, 4, and 6 months are shown. The numbers of subjects with food residues were 7, 7 and 7 for teeth, 8, 7 and 7 for the tongue, and 9, 7 and 7 for mucosa at 1, 4 and 6 months, respectively, after beginning professional care. The numbers of opportunistic pathogens were 2.8±1.8 and 3.2±1.3 for teeth, 3.6±1.3 and 3.7±0.9 on the tongue, and 3.4±1.2 and 3.2±1.1 on oral mucosal surfaces of elderly subjects with and without food residues, respectively, before the beginning of the study. Asterisks denote a significant difference in the chi-square test ($p < 0.05$, with food residues versus without food residues at 1, 4 or 6 months for each sample).

Discussion

We investigated the effects of routine oral hygiene using professional care in institutional elderly subjects determining the numbers of opportunistic pathogens in samples taken from the teeth, tongue, and mucosal surfaces. Our data show routine oral hygiene with professional care was effective in reducing infections by a number of different opportunistic pathogens on teeth surfaces when food residues were removed from patients during long-term care. In addition, short-term treatment of 1 month showed a significant reduction of opportunistic pathogens on the mucosal surfaces between elderly subjects with and without dentures. Dentures may be reservoirs of opportunistic pathogens as well as the teeth surfaces[17,18]; and may be a risk factor for opportunistic infection by many kinds of microorganisms in the institutionalized elderly. Therefore, dentures may disturb the effects of oral care on opportunistic pathogen infections in the short-term (1 month) on the oral mucosa. Consequently, long-term (4 and 6 months) oral care is necessary for decreasing the opportunistic pathogens in oral mucosa of elderly individuals with dentures.

It is important to consider the influence of oral health on elderly subjects living communally in the same institution, as well as communication between caregivers and those subjects. Accumulating evidence suggests community and health-care associated infections have a unique epidemiology; and the pathogens involved and outcomes may be related with nosocomial processes[19-21]. Further, transfers of microorganisms between the elderly or from caregivers and dental hygienists operating in the facility may have an influence on opportunistic infections in the oral cavity. Therefore, it may be possible that community- and care- associated infections were decreased or

abridged by controlling opportunistic pathogens in this long-term care study.

The oral biofilm is produced by the sequential attachment of bacteria; and is dependent on the various bacteria and the composition of the hard tissues involved[22-24]. Microorganisms become attached to and accumulate on surfaces of the oral cavity[25]. And the biofilm is known to be able to evade antimicrobial challenges from antibiotics or host immune defenses using multiple mechanisms[26-28] where antimicrobial agents fail to fully penetrate the bacterial cells that compose the biofilm[27]. Further, the bacterial community may increase on hard tissue surfaces presenting considerable hygiene and host-defense problems for elderly individuals[29]. Here our data shows remaining food residues made cleaning difficult to remove opportunistic pathogens from teeth surfaces in long-term routine care (Fig. 4A). Such food residues provide nutrition for microbial growth as well as a colonization site for biofilm formation. In a previous report on special oral care, the number of streptococci recovered shortly after treatment was reduced[30,31]. Using oral professionals to remove the biofilm and calculus and mouth washing with 0.5% povidone-iodine solution cleared the tooth and mucosal surfaces; and this then allowed re-establishment and growth by commensal bacteria such as streptococci that replace infections by opportunistic pathogens[31]. Professional oral care may be useful in elderly patients to prevent respiratory infections; however, routine oral care without professional care does not show a significant effect on the microbiological community of the oral cavity[32]. Therefore, we considered routine oral care with professional oral care cleaned the teeth and mucosal surfaces after which beneficial commensal bacteria re-established and grew dependent on the amount of the remaining food residues in the oral cavities of the elderly subjects. Thus, routine oral care over a long term that completely cleans the oral cavity may be necessary to remove biofilm and re-establish microbiological flora with the commensal bacteria.

In conclusion, routine oral cleaning along with professional care was able to control infection with many types of opportunistic pathogens on teeth and denture surfaces using long-term care of the institutionalized elderly but was not of value in short-term care; however, on the tongue and mucosal surfaces opportunistic pathogens were removed during the short term. Our data indicates the important role of routine oral care in cleaning hard and soft surfaces of the oral cavity where this improves the oral health for institutionalized elderly individuals.

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References

1. Senpuku H, Sogame A, Inoshita E, *et al.* N. Systemic diseases in association with microbial species in oral biofilm from elderly requiring care. Gerontology 2003;49:301-309.
2. Simons D, Kidd EA, Beighton D. Oral health of elderly occupants in residential homes. Lancet 1999;353:1761.
3. Yoneyama T, Yoshida M, Matsui T, *et al.* Oral care and pneumonia. Oral Care Working Group. Lancet 1999;354:515.
4. Pinto A, Yanai M, Nakagawa T, *et al.* Swallowing reflex in the night. Lancet 1994;344:820-821.
5. El-Solh AA, Pietrantoni C, Bhat A, *et al.* Microbiology of severe aspiration pneumonia in institutionalized elderly. Am J Respir Crit Care Med 2003;167:1650-1654.
7. El-Solh AA, Sikka P, Ramadan F, *et al.* Etiology of severe pneumonia in the very elderly. Am J Respir Crit Care Med 2001;163:645-651.
8. El-Solh AA, Aquilina AT, Dhillon RS, *et al.* Impact of invasive strategy on manegement of antimicrobial treatment failure in institutionalized older people with severe pneumonia. Am J Respir Crit Care Med 2002;166:1038-1043.
9. El-Solh AA, Pietrantoni C, Bhat A, *et al.* Colonization of dental plaques:a reservoir of respiratory pathogens for hospital-acquired pneumonia in institutionalized elders. Chest 2004;126:1575-1582.
10. Fourrier F, Duvivier B, Boutigny H, *et al.* Colonization of dental plaque:a source of nosocomial infections in intensive care unit patients. Crit Care Med 1998;26:301-308.
11. Terpenning MS, Taylor GW, Lopatin DE, *et al.* Aspiration pneumonia:dental and oral risk factors in an older veteran population. J Am Geriatr Soc 2001;49:557-563.
12. Mojon P, Budtz-Jorgenson E, Michel JP, *et al.* Oral health and history of respiratory tract infection in frail institutionalised elders. Gerodontology1997;14:9-16.
13. Yoneyama T, Yoshida M, Ohrui T, *et al.* Oral care reduces pneumonia in older patients in nursing homes. J Am Geriatr Soc 2002;50:430-433.
14. Yoneyama T, Hashimoto K, Fukuda H, *et al.* Oral hygiene reduces respiratory infections in elderly bed-bound nursing home patients. Arch Gerontol Geriatr 1996;22:11-9.
15. Salam MA, Senpuku H, Nomura Y, *et al.* Isolation of opportunistic pathogens in dental plaque, saliva and tonsil samples from elderly. Jpn J Infect Dis 2001;54:193-195.
16. Murray RGE, Brenner DJ, Bryant MR, *et al.* Bergey's Manual

of Systematic Bacteriology. Philadelphia, Lippincott, Williams & Wilkins, 1989, Vol1-4.
17. Marsh PD, Percival RS, Challacombe SJ. The influence of denture-wearing and age on the oral microflora. J Dent Res 1992;71:1374-1381.
18. Scannapieco FA. Pneumonia in nonambulatory patients. The role of oral bacteria and oral hygiene. J Am Dent Assoc 2006;137:21S-5S.
19. Tambyah PA, Habib AG. Ng TM, McGarry SA, *et al.* Community-acquired methicillin-resistant *Staphylococcus aureus* infection in Singapore is usually "Health care associated". Infect. Control Hosp Epidemiol 2003;24:436-438.
20. Friedoman ND, Kaye KS, Stout JE, *et al.* Health care-associated blood stream infections in adults. A reason to change the accepted definition of community-acquired infections. Ann Intern Med 2002;137:791-797.
21. Morin CA, Hadler JL. Population-based incidence and characteristics of community-onset *Staphylococcus aureus* infections with bacteremia in 4 metropolitan Connecticut areas. J Infect Dis 2001;184:1029-1034.
22. McEldowney S, Fletcher M. Adhesion of bacteria from mixed cell suspension to solid surfaces. Arch Microbiol 1987;148:57-62.
23. Loesche WJ, Syed SA, Laughon BE, *et al.* The bacteriology of acute necrotizing ulcerative gingivitis. J Periodontol 1982;53:223-230.
24. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbial Rev 1986;50:353-380.
25. Gibbons RJ. Role of adhesion in microbial colonization of host tissues. a contribution of oral microbiology. J Dent Res 1996;75:866-870.
26. Costerton JW, Stewart PS, Greeberg EP. Bacterial biofilms: a common cause of persistent infections. Science 1999;284: 1318-1322.
27. Millward TA, Wilson M. The effect of chlorhexidine on *Streptococcus sanguis* biofilms. Microbios 1989;58:155-164.
28. Wilson M. Susceptibility of oral bacterial biofilms to antimicrobial agents. J Med Microbiol 1996;44:79-87.
29. Percival RS, Challacombe SJ, Marsh PD. Age-related microbiological changes in the salivary and plaque microflora of healthy adults. J Med Microbiol, 1991;35:5-11.
30. Takeuchi H, Senpuku H, Matin K, *et al.* New dental drug delivery system for removing mutans streptococci from the oral cavity effect on oral micro flora. Jpn. J. Infect. Dis. 2000. 53:211-212.
31. Yoneda S, Imai S, Hanada N, *et al.* Effects of oral care on development of oral mucositis and Microorganisms in patients and Microorganisms in patients with esophageal cancer. Jpn. J. Infect. Dis. 2007;60:23-28.
32. Yoneyama T, Hashimoto K, Fukuda H, *et al.* Oral hygiene reduces respiratory infections in elderly bed-bound nursing home patients. Arch Gerontol Geriatr. 1996;22:11-9.

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ORIGINAL ARTICLE

Relation of bone turnover markers to periodontal disease and jaw bone morphology in elderly Japanese subjects

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OBJECTIVE: The purpose of this study was to evaluate the relation of bone turnover markers such as bone formation and resorption to periodontal disease and jaw bone morphology in elderly Japanese subjects.

SUBJECTS AND METHODS: We selected 148 subjects for participation in this study. All subjects were aged 77 years. The periodontal examination included the assessment of clinical attachment level (CAL). Biochemical parameters of bone turnover measured included urinary deoxypyridinoline, serum osteocalcin (S-OC), and serum bone-specific alkaline phosphatase. In addition, to evaluate the jawbone, we used the mandibular inferior cortex classification (MIC).

RESULTS: Serum osteocalcin had significantly higher (males: $P = 0.038$, females: $P = 0.041$) tendency for MIC Class (ANOVA). Multiple linear regression results showed that the number of remaining teeth and S-OC were negatively associated with the percentage of sites with ≥6 mm CAL ($R^2 = 0.322$, $P < 0.001$). Coefficients and betas were −0.71, −0.46 ($P < 0.001$) and −1.11, −0.28 ($P = 0.002$), respectively.

CONCLUSION: In conclusion, this study suggests that there is a significant relation of bone turnover markers to periodontal disease and jaw bone morphology in elderly Japanese subjects.

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Keywords: epidemiology; periodontal disease; bone turnover; jaw bone morphology

Introduction

Age-related physical disability and deterioration of physical function are becoming priorities in public health. Many functions of the human body decrease more rapidly from the age of 70 to 80 years than in earlier years. A turning point in the reduction of physical function also seems to occur during this period. In particular, muscle strength may decline greatly in subjects older than 75 years (Baumgartner *et al*, 1998; Morley *et al*, 2001).

Elderly people frequently experience periodontal disease (Slade and Spencer, 1995), characterized by absorption of alveolar bone and loss of soft-tissue attachment to teeth. Osteoporosis, which is characterized by low bone mass and micro-architectural deterioration of bone tissue, is the most common metabolic bone disease among the elderly (Prentice, 1997), and the incidence of osteoporotic fractures increases with age.

Because bone loss is a common feature of periodontal disease and osteoporosis, these diseases may share common etiologic factors that may affect the disease processes (Kribbs *et al*, 1990). We observed a significant correlation between skeletal bone mass measurements and the number of remaining teeth (Yoshihara *et al*, 2005). Other reports show that mandibular bone mass is significantly correlated with skeletal bone mass (Klemetti *et al*, 1993; von Wowern *et al*, 1994). Furthermore, bone mineral density of the mandible is affected by the mineral status of the skeleton and by any disease that causes generalized bone loss (Klemetti *et al*, 1993). Bone mineral density of the spine and leg is often used to evaluate bone condition. However, bone mineral density differs in different areas of the body. Many studies (Garnero *et al*, 1999, 2000; Chaki *et al*, 2000; Ross *et al*, 2000; Iki *et al*, 2004) have reported the efficacy of serum and urinary markers of bone turnover to evaluate bone metabolism. Low bone mass and architectural deterioration of bone tissue are caused by an imbalance of skeletal turnover maintained by the two opposite but normally balanced processes of bone formation and resorption (Rosen *et al*, 1997). Therefore, bone formation and bone resorption markers should be selected for evaluating bone metabolism. However, evidence from physiological and clinical studies is lacking, and data are often difficult to interpret because of potential size-confounding or bone remodeling transient effects.

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Serum bone-specific alkaline phosphatase (S-BAP), urinary deoxypyridinoline (U-DPD), and serum osteocalcin (S-OC) are often selected to measure bone turnover. S-BAP measures the enzymatic activity of osteoblastic cells (Stein *et al*, 1990). U-DPD is the non-reducible cross-link result from a posttranslational modification during the maturation of collagen (Egger *et al*, 1994). Osteocalcin is a calcium-binding protein of the bone and the abundant non-collagenous protein of the mineralized tissue (Lian and Gundberg, 1988).

On the other hand, Klemetti *et al* (1994) reported a new morphological classification of the mandibular inferior cortex. Several investigations have shown that the mandibular inferior cortex classification (MIC) may be a useful indicator of skeletal bone mineral density, the risk for osteoporotic fractures, or bone turnover (Klemetti and Kolmakow, 1997; Taguchi *et al*, 2003; Deguchi *et al*, 2008). Other reports have shown satisfactory levels of reproducibility of using MIC (Klemetti *et al*, 1994; Taguchi *et al*, 1996; Halling *et al*, 2005).

The purpose of this study was to evaluate the relation of bone turnover markers such as bone formation and resorption to periodontal disease and jaw bone morphology in elderly Japanese subjects.

Material and methods

Study population and clinical assessments

The population for this study was drawn from the Niigata study. Briefly, the Niigata study was a prospective community-based study that was initiated in 1998 to evaluate the relationship between an individual's general health status and his/her history of dental disease. Initially, questionnaires were sent to all inhabitants (n = 4542) aged 70 years based on a registry of residents in Niigata city in Japan; all recipients were informed of the purpose of this survey. Among those who were randomly selected to participate in the Niigata study (n = 600), 398 subjects who turned 70 in 1998, and were aged 77 years in 2005 underwent annual dental examinations. We selected 148 of these 398 subjects (79 males and 69 females) for participation in this study because they had one or more teeth, did not take medicine for bone disorders (tamoxifen, anabolic steroids, bisphosphonate, or estrogen), and did not have a diagnosis of fracture based on an X-ray assessment by medical doctors. All subjects were Japanese, in good general health, and did not require special care for their daily activities. Subjects were homogenous in terms of race, and we restricted the age to 77 years; this served to exclude the influence of race and age variations on results. The subjects for the study agreed to undergo medical and dental examinations, and signed informed consent forms regarding the protocol, which was reviewed and approved by the Ethics Committee of the Faculty of Dentistry, Niigata University.

The periodontal examination included the assessment of probing pocket depth (PPD) and clinical attachment level (CAL) at six sites around each tooth. Probing was performed using a pressure constant probe (Vivacare TPS Probe®; Vivacare, Schaan, Liechtenstein) at a probing force of 20 g and rounded to the nearest whole millimeter. The periodontal examination was carried out by four trained dentists under sufficient illumination using artificial light. Calibration of the examiners was carried out in volunteer patients at the Faculty Hospital. As determined by replicate examinations in 10 patients, the percent agreement (± 1 mm) ranged from 87.5% to 100% for PPD and from 83.3% to 100% for CAL. The κ ranged from 0.81 to 1.00 for PPD and from 0.74 to 1.00 for CAL.

We conducted personal interviews with subjects to obtain information regarding smoking habits. Urine was collected over 24 h (7:00 AM to 7:00 AM the day after the dental examination). On the day of urine collection, usual food and fluid intake were measured. The subject's blood was taken in the morning of the dental examination. Biochemical parameters of bone turnover were measured, including U-DPD (nM/nM*Cr) as bone resorption marker, S-OC (ng/ml) and S-BAP (U/l) as bone formation markers. U-DPD data were corrected by the urinary creatinine concentration measured by a standard colorimetric method. All laboratory tests were carried out at a commercial laboratory (BML, Inc, Tokyo, Japan).

All panoramic radiographs were obtained using SUPER VERAVIEW X-500 (Morita Co., Tokyo, Japan) at 5–10 mA and 15 sec; kV varied between 60 and 80. We used screens of speed group 200 (HG-M; Fuji Photo Film Co., Tokyo, Japan) and film (UR-2; Fuji Photo Film Co.). We used MIC to evaluate the jaw bone. The inferior cortex was detected on both sides of the mandible, distally from the mental foramen (Figure 1). Subjects were divided into three groups according to the following criteria: normal cortex (C1) – the endosteal margin of the cortex was even and sharp on both sides; mildly to moderately eroded cortex (C2) – the endosteal margin showed semilunar defects (lacunar resorption) or seemed to form endosteal cortical residues (1–3 layers) on one or both sides; and severely eroded cortex (C3) – the cortical residues were clearly porous. Dental panoramic radiographic measurements were estimated by a single examiner. The examiner had 4 years of experience in using MIC. Before we carried out this study, we measured the reproducibility of MIC by two observers including the examiner in this study. First, two observers (observer A and B) independently read 100 films. Observer A (the examiner in this study) read the 100 films again, with an interval of 2 weeks between his assessments. The intra- and inter-observer agreements on MCI were calculated as percentage and κ value. Overall agreements for intra- and inter-observer performances were 91.0% and 86.0%, respectively. The κ values for intra- and inter-observer agreement were 0.85 and 0.77, respectively.

Statistical analysis

Mean and standard deviations were used to characterize continuous variables. For descriptive data, the characteristics according to MIC of each gender were evaluated. We categorized subjects by tertiles according to the percentage of sites with ≥6 mm CAL (6+ mm CAL).