subjects 50-60 yrs old had moderate to advanced periodontitis. Accordingly, we used the top 20% for poor periodontal health. Subjects with means in the 30th percentile were assigned to the periodontally healthy group and the remaining 50% of the patients to the intermediate group.

The morning after subjects had fasted overnight, blood samples were collected from the antecubital vein and were analyzed according to previously described methods (Kubo *et al.*, 1999). The World Health Organization criteria for the diagnosis of diabetes were applied (Alberti and Zimmet, 1998): normal glucose tolerance (NGT, fasting and two-hour post-challenge plasma glucose levels < 110 mg/dL and < 140 mg/dL, respectively), diabetes (fasting or two-hour post-challenge plasma glucose levels ≥ 126 mg/dL or ≥ 200 mg/dL, respectively), and impaired glucose tolerance (IGT, all others with some glucose tolerance impairment including impaired fasting glucose, *i.e.*, with one of the two glucose tolerance levels between normal and diabetic values and the other below the diabetic level).

Glucose intolerance was defined as developing in subjects who had NGT in 1988, but had IGT or were diabetic in 1998. For 597 subjects, HbA1c data for both 1988 and 1998 were available. The change in HbA1c was defined as the 1998 value minus the 1988 value. An increase in HbA1c of ≥ 0.2%, which corresponded to the proportion of subjects in whom there was deterioration in OGTT, was considered progression. Progression (yes/no) in HbA1c served as the dependent variable in the logistic regression analysis. Each subject completed a self-administered questionnaire, which was checked by trained nurses. Age (continuous), sex, BMI (continuous), exercise frequency (0, 1-2, ≥ 3 times a wk), alcohol consumption (converted to 100% ethanol *per* month: 0, 1-399, 400-1199, ≥ 1200 g), and smoking habits (never, past, current smoker) were used as independent variables, all having been reported risk factors for type 2 diabetes in multivariate analyses (Hu *et al.*, 2001).

The differences in the mean values were evaluated by Student's *t* test and Welch's *t* test in the case of unequal variances. To protect against spurious significance among multiple inferences, we used Bonferroni's correction to interpret the significance of the *p*-values.**(AQ)** Differences in percentages were evaluated by Pearson's correlation, and trends were evaluated by the Mantel-Haenszel chi-squared test. We performed multivariate logistic regression analyses to determine the effect of periodontal condition on the glucose tolerance status, and calculated the odds ratio (OR) and 95% confidence interval (CI). SPSS version 11.0 (SPSS Japan Inc., Tokyo, Japan) was used for the analyses. The design of the study and procedures for obtaining informed consent were approved by the Ethics Committee of Kyushu University Faculty of Dental Science and the Department of Health and Welfare of Hisayama town.

RESULTS

In 1998, 191 of 961 subjects had IGT, and 101 had diabetes, based on the results of the OGTT. The characteristics of subjects with NGT, IGT, and diabetes were compared (Table 1). Periodontal condition and many of the other values were worse in subjects with poorer OGTT results. The numbers of subjects with NGT, IGT, and diabetes were compared according to periodontal condition by mean pocket depth and mean attachment loss (Table 1). The proportion of subjects with IGT and that with diabetes increased significantly with mean pocket depth ($P = 0.0001$). The proportion with diabetes increased with mean attachment loss, while the proportion with IGT did not. The increased proportion of subjects with IGT and with diabetes, according to mean pocket depth, was recognized in the univariate and multivariate analyses, as shown in Table 2. In the highest category of pocket depth, the adjusted OR for IGT to NGT was 1.8 (95% CI, 1.1-2.9) as compared with the lowest category of pocket depth, adjusted for age, sex, BMI, exercise frequency, alcohol consumption, and smoking habits (Table 2). The adjusted OR for diabetes in the intermediate and high categories of pocket depth was 1.9 (95% CI, 1.0-3.4) and 2.6 (95% CI, 1.3-5.0), respectively. While there was a significant relationship between diabetes and mean attachment loss in both the univariate and multivariate analyses, we could not find any significant relationship between IGT and mean attachment loss (Table 2).

The OGTT results were analyzed retrospectively for 591 subjects (Table 3). Of these, 415 subjects (70.2%) had NGT and 176 subjects (29.8%) had IGT or diabetes in 1988, and the

Table 2. Relationship between Periodontal Condition and Glucose Tolerance Status in 1998

	Subjects with NGT and with IGT, n = 860						Subjects with NGT and with Diabetes, n = 770					
			Univariate Analysis		Multivariate Analysis[a]				Univariate Analysis		Multivariate Analysis[a]	
Periodontal Condition	NGT	IGT	P[b]	P[c]	OR (95% CI)	P	NGT	Diabetes	P[b]	P[c]	OR (95% CI)	P
Mean pocket depth (mm)	No. of subjects (%)		0.006	0.001			No. of subjects (%)		0.0002	< 0.0001		
Low (< 1.3)	230 (83.0)	47 (17.0)			1		230 (92.7)	18 (7.3)			1	
Intermediate (1.3-2.0)	322 (77.4)	94 (22.6)			1.4 (0.9-2.0)	0.13	322 (86.3)	51 (13.7)			1.9 (1.0-3.4)	0.037
High (> 2.0)	117 (70.1)	50 (29.9)			1.8 (1.1-2.9)	0.013	117 (78.5)	32 (21.5)			2.6 (1.3-5.0)	0.004
Mean attachment loss (mm)			0.13	0.60					< 0.0001	< 0.0001		
Low (< 1.5)	184 (76.3)	57 (23.7)			1		184 (91.1)	18 (8.9)			1	
Intermediate (1.5-2.5)	366 (80.3)	90 (19.7)			0.8 (0.5-1.1)	0.16	366 (89.1)	45 (10.9)			1.1 (0.6-2.0)	0.83
High (> 2.5)	119 (73.0)	44 (27.0)			1.0 (0.6-1.7)	0.97	119 (75.8)	38 (24.2)			2.0 (1.0-3.9)	0.038

[a] Logistic regression analysis adjusted for age (continuous), sex, BMI (continuous), exercise frequency (0, 1-2, ≥ 3 times a wk), alcohol consumption (converted to 100% ethanol *per* month; 0, 1-399, 400-1199, ≥ 1200 g), and smoking habits (never, past, current smoker).
[b] P value for the non-linear component calculated by Pearson's chi-square test.
[c] P value for the linear component calculated by the Mantel-Haenszel chi-square test.

Table 3. Relationship between Periodontal Condition in 1998 and Glucose Tolerance Status in 1988 and 1998

	All Subjects in 1988, n = 591 OGTT in 1988				All Subjects in 1988, n = 591 OGTT in 1998				Limited to Subjects with NGT in 1988, n = 415 OGTT in 1998			
Periodontal Condition	NGT	IGT and Diabetes	P[a]	P[b]	NGT	IGT and Diabetes	P[a]	P[b]	NGT	IGT and Diabetes	P[a]	P[b]
Mean pocket depth (mm)	No. of subjects (%)		0.039	0.016	No. of subjects (%)		0.0001 < 0.0001		No. of subjects (%)		0.003	0.0007
Low (< 1.3)	123 (77.8)	35 (22.2)			126 (79.7)	32 (20.3)			110 (89.4)	13 (10.6)		
Intermediate (1.3-2.0)	202 (68.5)	93 (31.5)			193 (65.4)	102 (34.6)			160 (79.2)	42 (20.8)		
High (> 2.0)	90 (65.2)	48 (34.8)			79 (57.2)	59 (42.8)			64 (71.1)	26 (28.9)		
Mean attachment loss (mm)			0.12	0.038			0.11	0.053			0.23	0.16
Low (< 1.5)	97 (75.8)	31 (24.2)			91 (71.1)	37 (28.9)			80 (82.5)	17 (17.5)		
Intermediate (1.5-2.5)	220 (70.7)	91 (29.3)			215 (69.1)	96 (30.9)			181 (82.3)	39 (17.7)		
High (> 2.5)	98 (64.5)	54 (35.5)			92 (60.5)	60 (39.5)			73 (74.5)	25 (25.5)		
Total	415 (70.2)	176 (29.8)			398 (67.3)	193 (32.7)			334 (80.5)	81 (19.5)		

[a] P value for the non-linear component was calculated by Pearson's chi-square test.
[b] P value for the linear component was calculated by the Mantel-Haenszel chi-square test.

proportions with IGT and diabetes increased slightly in 1998 (32.7%). The proportion with IGT and diabetes in 1988 (Table 3, left column) was greater in deeper pocket depth categories (P for trend = 0.016). The relationship between pocket depth and the proportion with IGT and diabetes was stronger in 1998 (P for trend < 0.0001) than in 1988 (Table 3).

Of the 415 subjects with NGT in 1988, 81 subjects (19.5%) had IGT or diabetes in 1998. The proportion with IGT and of diabetic subjects who had NGT in 1988 increased with mean pocket depth (P for trend = 0.0007; Table 3, right column), more than was the case for all of the subjects (center column). Of the 176 subjects with IGT or diabetes in 1988, there were 64 subjects (36.4%) whose glucose tolerance condition had converted to NGT (data not shown). The proportion of these subjects was larger in subjects with a lower mean pocket depth than in those with deeper pockets, but the trend did not reach statistical significance (P = 0.19). The relationship between attachment loss and OGTT results in 1998 was not significant.

Table 4 compares the proportions with IGT with those with NGT and those with diabetes with those with NGT separately for the same 415 subjects with NGT in 1988. The proportion of subjects with IGT increased more in those with deeper pockets (P for trend = 0.001). The multivariate logistic regression analysis, with the deterioration in glucose tolerance over the 10-year interval as the dependent variable (yes/no), shows that the intermediate and high categories of mean pocket depth were significantly associated with a deterioration in the OGTT from NGT to IGT, as compared with the deterioration of those in the lowest category of mean pocket depth (Table 4). The OR was greater in subjects with deeper periodontal pockets (P for trend = 0.018 in the multivariate analysis). Mean attachment loss was analyzed in the same manner, but no significant association was observed (Table 4). The OR for diabetes also increased with pocket depth, but did not reach statistical significance (Table 4) due to the small sample size and corresponding lack of power.

Of the 597 subjects for whom HbA1c data were available in both 1988 and 1998, each additional millimeter in mean pocket depth corresponded to a 0.13% increase in HbA1c, according to a multivariate linear regression (P = 0.007). Excluding the 52 subjects diagnosed with diabetes in 1988, the subjects in the high category of pocket depth had a significant increase in HbA1c of $\geq$ 0.2% from 1988 to 1998 (18% of the 545 non-diabetic subjects), as compared with subjects in the low category, according to a similar multivariate analysis (OR, 2.4; 95% CI, 1.2-4.6; P = 0.009). The mean attachment loss was not associated with an increase in HbA1c according to a similar analysis.

DISCUSSION

In a cross-sectional analysis of the 1998 data, deep periodontal pockets were significantly associated with IGT and diabetes, whether in univariate or multivariate models controlling for known risk factors for diabetes. Since worsening of the diabetic condition is associated with deteriorating periodontal tissue (Page *et al.*, 1997), a cross-sectional relationship between deep pockets and diabetes was presumed. Although no increased risk of periodontal disease with IGT has been reported, our results suggest the hypothesis that not only diabetes but also IGT increases the risk of deeper pockets. Considering the possible effects of diabetic and IGT conditions on periodontal tissue, we excluded subjects with diabetes and with IGT in 1988 retrospectively. As expected, the OR for periodontal condition and the development of glucose intolerance from NGT in 1988 to IGT in 1998 (Table 4) was higher and the p-value lower than in the cross-sectional analysis (Table 2), although there were fewer subjects. Moreover, the relationship between pocket depth and glucose intolerance in 1998 was stronger than the relationship between pocket depth and glucose intolerance in 1988 (Table 3). These results indicate that deep pockets were more closely associated with the development of glucose intolerance from normal status than the past glucose tolerance status itself. About one-third of the subjects with IGT or diabetes in 1988 improved their glucose status to NGT in 1998. In this subgroup, the proportion with NGT in 1998 was higher

Table 4. Relationship between Periodontal Condition and Glucose Tolerance Status in 1998, Limited to the Subjects with Normal Glucose Tolerance in 1988

	Subjects with NGT and with IGT in 1998, n = 406						Subjects with NGT and with Diabetes in 1998, n = 343					
			Univariate Analysis		Multivariate Analysis[a]				Univariate Analysis		Multivariate Analysis[a]	
Periodontal Condition	NGT	IGT	P[b]	P[c]	OR (95% CI)	P	NGT	Diabetes	P[b]	P[c]	OR (95% CI)	P
Mean pocket depth (mm)	*No. of subjects (%)*		0.005	0.001			*No. of subjects (%)*		0.37	0.30		
Low (< 1.3)	110 (90.2)	12 (9.8)			1		110 (99.1)	1 (0.9)			1	
Intermediate (1.3-2.0)	160 (81.6)	36 (18.4)			2.1 (1.0-4.2)	0.048	160 (96.4)	6 (3.6)			5.0 (0.6-45)	0.15
High (> 2.0)	64 (72.7)	24 (27.3)			3.1 (1.4-6.9)	0.005	64 (97.0)	2 (3.0)			3.7 (0.3-47)	0.31
Mean attachment loss (mm)			0.28	0.24					0.57	0.29		
Low (< 1.5)	80 (83.3)	16 (16.7)			1		80 (98.8)	1 (1.2)			1	
Intermediate (1.5-2.5)	181 (84.2)	34 (15.8)			0.9 (0.4-1.7)	0.66	181 (97.3)	5 (2.7)			3.6 (0.4-34)	0.27
High (> 2.5)	73 (76.8)	22 (23.2)			1.3 (0.6-2.9)	0.49	73 (96.1)	3 (3.9)			4.8 (0.4-55)	0.21

[a] Logistic regression analysis adjusted for age (continuous), sex, BMI (continuous), exercise frequency (0, 1-2, ≥ 3 times a wk), alcohol consumption (converted to 100% ethanol per month; 0, 1-399, 400-1199, ≥ 1200 g), and smoking habits (never, past, current smoker).
[b] P value for the non-linear component calculated by Pearson's chi-square test.
[c] P value for the linear component calculated by the Mantel-Haenszel chi-square test.

in subjects with shallower pocket depths than in those with deeper pocket depths, although it did not reach statistical significance (data not shown). This may relate to previous reports that periodontal treatment has beneficial effects on glucose control in diabetics.

The OR for diabetes from NGT in the past 10 years was not significant, since there were only nine diabetic subjects (Table 4). The analyses of HbA1c over the 10-year period showed that it increased more in subjects with deep periodontal pockets, supporting the results of the OGTT. In all the analyses, severe attachment loss was not associated with IGT, although it was significantly associated with diabetes in cross-sectional analyses, as is well-known (Page *et al.*, 1997). Since attachment loss usually means gingival recession plus periodontal pockets, such patients are less likely to harbor subgingival bacteria than those with deep periodontal pockets. Generally, periodontal pockets are directly related to subgingival bacteria, while attachment loss is not. From these results, especially from the retrospective analyses, chronic inflammation from subgingival pathogens in deep periodontal pockets may affect glucose control in non-diabetic subjects.

Tumor necrosis factor alpha (TNF-α), which is produced from the increased amounts of adipose tissue in obese subjects, plays a predominant role in insulin resistance (Uysal *et al.*, 1997; Zinman *et al.*, 1999). In the periodontium, it is well-known that the lipopolysaccharides continuously provided by Gram-negative bacteria, such as *P. gingivalis*, trigger the production of TNF-α, a pro-inflammatory cytokine (Page *et al.*, 1997). The area of the interface where these subgingival bacteria can interact with gingival tissue is estimated to be as much as 72 cm^2 in patients with severe periodontitis and many deep pockets; this results in an enormous burden of Gram-negative bacteria (Page *et al.*, 1997). Periodontal treatment to remove these bacteria appears to reduce circulating TNF-α levels (Iwamoto *et al.*, 2001). TNF-α induced from periodontal pathogens may increase insulin resistance, which may lead to a risk of cardiovascular disease (Genco *et al.*, 2002).

There are several potential limitations to our findings. The participation rates by residents in the health and periodontal examinations were 52% and 26%, respectively. Subjects with fewer than 10 teeth were excluded. We had no data on subjects' periodontal condition in 1988. According to NHANES III, mean pocket depth was about the same in every age group, and the percentage of sites with deep pockets was the same in subjects over 50 yrs old, while attachment loss increased with age (Brown *et al.*, 1996; Albandar *et al.*, 1999). Indeed, in our subjects, the correlation coefficient between mean pocket depth and age was smaller ($r = 0.13$) than that between mean attachment loss and age ($r = 0.28$). These observations support the assumption that the periodontal pockets of the subjects examined in 1998 might not have changed much in the previous 10 years. Based on this assumption, we considered the possibility that periodontal disease had an adverse effect on glucose tolerance. Owing to its cross-sectional character, based on a 1998 examination with additional 1988 OGTT data, the study cannot provide a clear answer to the question of whether having deep pockets is the cause or the result of IGT. We can conclude that deep pockets and current glucose tolerance status, such as IGT and diabetes, are significantly associated. The significant relationship between deep pockets and the past development of glucose intolerance in non-diabetics suggests that periodontal disease is a risk factor for type 2 diabetes. Prospective cohort studies with sufficient subjects are required to confirm this suggestion.

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Relationship Between Electrocardiographic Abnormalities and Periodontal Disease: The Hisayama Study

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Background: Recent studies have suggested a relationship between periodontitis and cardiovascular disease (CVD). This study investigated the relationship between periodontitis and electrocardiographic (ECG) abnormalities, which are known predictors of CVD.

Methods: We examined the periodontal status of 1,111 residents of Hisayama Town, Fukuoka, Japan. Nine hundred fifty-seven (957) subjects (374 males, 583 females) with ≥10 teeth and without a medical history of CVD were included in the analysis. Probing depth (PD) and clinical attachment level (CAL) were measured on two randomly selected quadrants, one maxillary and one mandibular. A 12-lead ECG was recorded using a standard electrocardiograph. ECG abnormalities included left ventricular hypertrophy (Minnesota code 3-1) and ST depression (4-1, 2, 3). The relation of periodontal condition and ECG abnormalities was assessed with logistic regression analysis.

Results: Univariate analysis revealed that mean probing depth, mean attachment loss, number of teeth, and plaque index were significantly associated with ECG abnormalities, as well as with known risk factors of CVD. In multivariate analysis, the subjects with deep pockets (mean probing depth ≥2 mm) had an increased risk for ECG abnormalities (odds ratio [OR] = 1.6; 95% confidence interval [CI] = 1.01 to 2.50) compared to the subjects with mean PD <2 mm. Subjects with severe attachment loss (mean CAL ≥2.5 mm) had also significant risk for ECG abnormalities (OR = 1.7; 95% CI = 1.07 to 2.67) compared to those whose mean CAL was <2.5 mm.

Conclusion: This study clearly shows the relationship between periodontitis and ECG abnormalities, which are important predictors of CVD. *J Periodontol 2004;75:791-797.*

KEY WORDS

Coronary disease/epidemiology; electrocardiography/abnormalities; periodontitis/complications; risk factors.

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Accumulated epidemiologic and laboratory evidence suggests that periodontal infection is a contributing risk factor for cardiovascular disease (CVD), a serious disease often leading to death.[1-6] Inflammation due to infection by several microorganisms, such as *Chlamydia pneumoniae, Helicobacter pylori,* and cytomegalovirus, has been implicated in the etiology of atherosclerosis, which is intrinsic to CVD.[7,8] Periodontitis is a chronic inflammatory disease caused by Gram-negative anaerobic bacteria, such as *Porphyromonas gingivalis* and *Tannerella forsythensis,* and is most prevalent in adults. Periodontal pathogens exist in atherosclerotic plaques, where they may play a role in the development and progression of atherosclerosis, suggesting a direct influence of periodontal pathogens on CVD.[9,10] However, some studies show no or at most a weak relationship between periodontitis and CVD.[11-14] Therefore, further evidence is required to clarify this relationship.[15]

Electrocardiographic (ECG) abnormalities are significantly related to subsequent death from coronary heart disease (CHD),[16] and are one of the most sensitive predictors of fatal CHD.[17] In particular, left ventricular hypertrophy (LVH) and ST depression are thought to be important predictors of heart disease and death from coronary events.[18-25] As ECG examinations take only a few minutes, cause no discomfort, and do not require the presence of a physician, they are widely used to screen for heart disease

in health examinations in Japan. An examination of the relationship between periodontitis and ECG abnormalities may produce detailed information on the effect of periodontitis on conditions preexisting CVD. This study examined the relationship between periodontal condition and ECG abnormalities in subjects without a medical history of CVD who underwent a health examination as part of the Hisayama Study.[26]

MATERIALS AND METHODS

Study Population

Hisayama is a subrural town that is adjacent to Fukuoka City, on Kyushu Island, in southern Japan. The population of the town has been approximately 7,000 for the past 30 years. Between July and September 1998, 2,180 residents (adult population between 20 and 81 years of age, mean age 55.8 ± 9.6 convenience sample) underwent a comprehensive health examination. We examined only the 1,111 dentate subjects, who received both oral and general examinations. The remaining 1,069 adults were either edentulous or refused an oral examination. Exclusion criteria included individuals with <10 teeth (because of the inherent difficulties in properly assessing periodontal health) and those with a medical history of CVD. Consequently, 957 individuals (374 males, 583 females) with ≥10 teeth and without a medical history of CVD were included. The design of the study, its data collection methods, and the procedures used to obtain informed consent were approved by the Department of General Affairs and Health and Welfare of Hisayama.

Oral Examination

The periodontal examination was performed on two randomly selected quadrants, one maxillary and one mandibular, following the method of the National Health and Nutrition Examination Survey III (NHANES III).[27] The periodontal examination was carried out by one of four dentists trained to perform a clinical examination of oral health status. The examiner reliability of the periodontal examination was verified by an interexaminer calibration of outpatients visiting Kyushu University Dental Hospital; the kappa value for the periodontal examination exceeded 0.8, suggesting very good inter-examiner agreement. Probing depth (PD) and clinical attachment level (CAL) were measured at mesio-buccal and mid-buccal sites for all of the teeth present in the two quadrants. Each subject's periodontal condition was classified according to their mean PD: ≥2 mm (20.1%) or <2 mm, and mean CAL: ≥2.5 mm (20.4%) or <2.5 mm. NHANES III found that about 20% of 50- to 60-year-old subjects had moderate to advanced periodontitis.[28] Accordingly, we used the 20th percentile to indicate poor periodontal conditions. Other oral examinations were performed following the methods recommended by the World Health Organization (WHO). Oral hygiene status was evaluated using a plaque index.[29]

General Examination

A 12-lead ECG was recorded in the supine position using a standard electrocardiograph. Using the Minnesota code[30] to evaluate the ECG, we defined LVH (Minnesota code 3-1) and ST-segment depression (4-1, 2, 3) as ECG abnormalities, both of which are considered important predictors of heart disease and death from coronary events.[18-25] Blood pressure was measured three consecutive times, after resting for at least 5 minutes, using a standard mercury sphygmomanometer with the subjects in the sitting position and the average value was used for the analysis. Blood samples were collected from an antecubital vein after an overnight fast. The laboratory analyses of the blood samples followed previously described methods.[26] The subjects were evaluated with a 75 g oral glucose tolerance test and categorized into three groups: normal (fasting and 2-hour post-challenged plasma glucose levels <110 and <140 mg/dl, respectively), diabetic (fasting or 2-hour post-challenged plasma glucose levels ≥126 or ≥200 mg/dl, respectively), and impaired (other than the above). The body-mass index (BMI) was defined as the weight in kilograms divided by the square of the height in meters. Each subject completed a self-administrated questionnaire in advance, which was checked by trained nurses. The questionnaire included social status, exercise frequency (0, 1-2, ≥3 times a week), alcohol consumption (converted to 100% ethanol per month; 0, 1-399, 400-1199, ≥1200 g), and smoking status (never, former, current smoker).

Statistical Analysis

The subjects' characteristics were compared statistically using the *t* test for quantitative data and the chi-square test for the ratio of categorical variables between subjects with and without ECG abnormalities. We recognized a significant relationship ($P<0.001$) between the periodontal parameters as an independent variable and ECG abnormalities as the dependent variable in a univariate logistic regression analysis. Subsequently, we added the variables that were significantly related to ECG abnormalities in the univariate analysis to a bivariate logistic regression analysis with the periodontal parameter and evaluated them individually for both confounding and statistical significance. The variables that showed the statistical significance in the bivariate analysis with the periodontal parameters were entered into a multivariate logistic regression analysis to calculate the odds ratio (OR) and 95% confidence interval (CI) for ECG abnormalities as the dependent variable. All analyses were performed using a software program.§

§ SPSS Version 6.1, SPSS Japan Inc., Tokyo, Japan.

RESULTS

Table 1 shows the relationship between ECG abnormalities and each study variable. The variables that were significantly associated with ECG abnormalities in the univariate analysis were age, number of teeth, systolic blood pressure, diastolic blood pressure, mean PD, mean CAL, gender, plaque index, smoking status, alcohol consumption, and exercise frequency. There were 147 subjects who had both a mean PD ≥2 mm and a mean CAL ≥2.5 mm (70.3% of the subjects who had a mean PD ≥2 mm and 67.7% of the subjects who had a mean CAL ≥2.5 mm). Since the mean PD and mean CAL, which represent the subjects' periodontal condition, were closely related ($r = 0.78$, $P < 0.001$), these two variables were analyzed separately in the following analyses. Variables that were significant in the univariate analysis were subjected to a bivariate logistic regression analysis with the periodontal parameters. Age, gender, number of teeth, systolic blood pressure, diastolic blood pressure, smoking status, alcohol consumption, and exercise frequency were the significant variables in the analysis when considered with each of the two periodontal parameters (mean PD and mean CAL). All of the significant variables and the periodontal parameters were then subjected to a multivariate logistic regression analysis. We used systolic blood pressure as the representative blood pressure. As a result, mean PD, mean CAL, age, and systolic blood pressure were identified as significant risk factors for ECG abnormalities (Tables 2 and 3). The results for mean PD and mean CAL were similar, and the OR for ECG abnormalities for the subjects whose mean PD was ≥2 mm was 1.6 compared to the subjects with

Table 1.

Population Characteristics of Subjects According to ECG Status

	ECG Abnormalities		
	Negative (N = 832)	Positive (N = 125)	
	Mean ± SD		*P* Value*
Age (years)	55.1 ± 9.6	60.2 ± 8.9	<0.001
Teeth (N)	25.4 ± 3.9	23.9 ± 4.2	<0.001
Decayed teeth (N)	0.7 ± 1.4	0.7 ± 1.5	NS
Body-mass index (kg/m^2)	23.2 ± 3.3	22.8 ± 3.0	NS
Systolic blood pressure (mm Hg)	126.6 ± 19.1	142.9 ± 24.7	<0.001
Diastolic blood pressure (mm Hg)	77.5 ± 10.8	82.7 ± 10.5	<0.001
Triglyceride (mg/dl)	124.5 ± 85.5	138.4 ± 97.3	NS
HDL cholesterol (mg/dl)	58.3 ± 13.7	56.0 ± 13.1	NS
LDL cholesterol (mg/dl)	123.2 ± 32.3	117.7 ± 35.9	NS
Mean PD (mm)	1.6 ± 0.5	1.8 ± 0.6	<0.001
Mean CAL (mm)	1.9 ± 0.8	2.2 ± 0.8	<0.001
	N (%)		*P* Value†
Gender			
Male	311 (83.2)	63 (16.8)	<0.01
Female	521 (89.4)	62 (10.6)	
Mean PD			
<2 mm	665 (88.9)	83 (11.1)	<0.01
≥2 mm	167 (79.9)	42 (20.1)	
Mean CAL			
<2.5 mm	662 (89.5)	78 (10.5)	<0.001
≥2.5 mm	170 (78.3)	47 (21.7)	
Plaque index			
Low	404 (90.2)	44 (9.8)	<0.01
High	428 (84.1)	81 (15.9)	
Social class			
Managerial position	50 (89.3)	6 (10.7)	NS
Office worker	446 (88.1)	60 (11.9)	
Primary industries	58 (76.3)	18 (23.7)	
Factory worker	77 (80.2)	19 (19.8)	
Housewife or without job	201 (90.1)	22 (9.9)	
Smoking status			
Never	568 (88.9)	71 (11.1)	<0.05
Former	111 (81.6)	25 (18.4)	
Current	153 (84.1)	29 (15.9)	
Alcohol consumption (converted to 100% ethanol per month)			
0-399 g	647 (88.6)	83 (11.4)	<0.01
400-1199 g	114 (85.1)	20 (14.9)	
≥1200 g	71 (76.3)	22 (23.7)	

Table 1. (continued)

Population Characteristics of Subjects According to ECG Status

	ECG abnormalities		
	Negative (N = 832)	Positive (N = 125)	P Value†
Exercise frequency			
0-2 times a week	595 (85.5)	101 (14.5)	<0.05
≥3 times a week	237 (90.8)	24 (9.2)	
Glucose tolerance‡			
Normal	587 (87.9)	81 (12.1)	NS
Impaired	159 (85.5)	27 (14.5)	
Diabetic	77 (82.8)	16 (17.2)	

* *t* test.
† Chi-square test.
‡ The 10 patients who underwent glucose testing were excluded.

Table 2.

A Multiple Logistic Regression Analysis of the Effect of the Explanatory Variables Including Mean PD on ECG Abnormalities

	Dependent Variable = ECG Abnormalities (negative = 0, positive = 1)				
Independent Variable	B	SE	P Value	OR	95% CI
Age	0.033	0.013	0.011	1.0	1.01 – 1.06
Gender (male = 0, female = 1)	–0.076	0.324	0.814	0.9	0.49 – 1.75
Mean PD (<2 mm = 0, ≥2 mm = 1)	0.463	0.231	0.045	1.6	1.01 – 2.50
N teeth	–0.023	0.026	0.383	1.0	0.93 – 1.03
Systolic blood pressure	0.028	0.005	<0.001	1.0	1.02 – 1.04
Smoking status					
Never				1.0	
Former	0.122	0.349	0.726	1.1	0.57 – 2.24
Current	0.205	0.330	0.534	1.2	0.64 – 2.35
Alcohol consumption					
0 – 399 g				1.0	
400 – 1199 g	0.133	0.320	0.678	1.1	0.61 – 2.14
≥1200 g	0.323	0.347	0.352	1.4	0.70 – 2.73
Exercise frequency (0-2 times a week = 0, ≥3 times a week = 1)	–0.439	0.254	0.084	0.6	0.39 – 1.06

a mean PD <2 mm (Table 2); the OR for the subjects with a mean CAL ≥2.5 mm was 1.7 compared with the subjects with a mean CAL <2.5 mm (Table 3). Older age and higher systolic blood pressure increased the risk of ECG abnormalities significantly. Exercise frequency did not reach statistical significance ($P = 0.084$, Table 2; $P = 0.069$, Table 3).

DISCUSSION

Resting ECG abnormalities were significant predictors of both total and fatal CHD.[17] Three Chicago epidemiological studies found that men with major ECG abnormalities had considerably higher death rates than those with a normal ECG, and in a multivariate analysis ECG abnormalities had a significant relationship to death from CHD and to death from all causes, independent of other confounding variables.[16] Therefore, ECG examinations are widely used to screen for heart disease in health examinations. We defined LVH and ST depression as ECG abnormalities using the Minnesota code, which has been used in many epidemiological studies. LVH suggesting hypertensive heart disease is one of the most frequent ECG findings in the asymptomatic general population in Japan and is considered a predictor of future atherosclerotic disease.[21,23] LVH was reported to be one of the predictors of sudden cardiac death among Hawaiian-Japanese men.[22] The Framingham study indicated that patients with LVH by ECG had a greater risk of sudden death and acute myocardial infarction than subjects with normal hearts.[19] In addition, ST depression suggesting ischemia has been reported to be an important predictor of CVD.[24,25] In healthy subjects, ST depressions in an ECG using the Minnesota code is associated with increased risk of coronary events.[18,20] De Bacquer et al. reported that both ST segment depression and LVH were the most predictive ECG findings for CVD death.[25] Considering these reports, the relationship between periodontitis and ECG abnormalities observed in this study suggests a relationship between periodontitis and CVD.

Table 3.

Multiple Logistic Regression Analysis of the Effect of the Explanatory Variables Including Mean CAL on ECG Abnormalities

	Dependent Variable = ECG Abnormalities (negative = 0, positive = 1)				
Independent Variable	B	SE	*P* Value	OR	95% CI
Age	0.030	0.013	0.021	1.0	1.00 – 1.06
Gender (male = 0, female = 1)	–0.110	0.324	0.735	0.9	0.47 – 1.69
Mean CAL (<2.5 mm = 0, ≥2.5 mm = 1)	0.523	0.234	0.025	1.7	1.07 – 2.67
N teeth	–0.019	0.026	0.470	1.0	0.93 – 1.03
Systolic blood pressure	0.028	0.005	<0.001	1.0	1.02 – 1.04
Smoking status					
Never				1.0	
Former	0.098	0.352	0.780	1.1	0.55 – 2.20
Current	0.138	0.337	0.682	1.1	0.59 – 2.22
Alcohol consumption					
0 – 399 g				1.0	
400 – 1199 g	0.145	0.321	0.653	1.2	0.62 – 2.17
≥1200 g	0.303	0.347	0.382	1.4	0.69 – 2.67
Exercise frequency (0-2 times a week = 0, ≥3 times a week = 1)	–0.464	0.255	0.069	0.6	0.38 – 1.04

Periodontal disease is a chronic inflammatory disease caused by Gram-negative anaerobic bacteria. Reports that periodontitis results in higher systemic levels of C-reactive protein (CRP), interleukin-6, and neutrophils suggest that elevated levels of these inflammatory substances cause inflammatory changes to atherosclerotic lesions, increasing the risk of cardiac or cerebrovascular events.[31,32] Periodontal pathogens, such as *Tannerella forsythensis, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Prevotella intermedia,* and *Streptococcus sanguis*, have been detected in atherosclerotic plaques and implicated in the infectious etiology of atherosclerosis.[9,10] Herzberg and Meyer showed that *S. sanguis* contributed to acute thrombotic events in experimental rabbits;[33,34] *P. gingivalis* vesicles on the outer membrane have been reported to possess platelet aggregation activity.[35] These studies suggest potential causal mechanisms that may underlie the relationship between periodontitis and CVD.

Although several studies have reported no relationship between periodontitis and CVD,[11,12,14] these studies had limitations in the way they evaluated periodontal conditions. Howell et al. used a self-reported periodontal evaluation,[11] which is far from accurate compared with direct measurement using a periodontal probe. Genco et al. commented that the Russell periodontal index used in the National Health and Nutrition Examination Survey I[12] is subjective and less accurate than objective periodontal measurement and is thought to result in the misdiagnosis of periodontal disease.[36] In another study, Hujoel et al. defined edentulous status as completely eliminating dental infections, and showed that edentulous subjects did not have a lower CVD risk than subjects with periodontitis.[13] Both edentulous status and severe gingivitis are significant risks for CVD;[37] therefore, methods that compare edentulous status with periodontitis may not be appropriate. Indeed, both edentulous and periodontitis subjects have increased levels of serum CRP, which is a risk factor for CVD.[38] The relationship between edentulous status and increased risk of CVD is still unresolved.

We examined only the mesiobuccal and mid-buccal sites of all of the teeth in two randomly selected quadrants. However, Papapanou et al. reported that full mouth and partial estimates of the extent and severity of CAL were strongly correlated, and, in particular, the severity index (mean value of the sites with >1 mm CAL) was highly correlated ($r = 0.93$) in the 55- to 64-year-old age group.[39] We excluded subjects with <10 teeth from our study because of the inherent difficulties in properly assessing periodontal health in these patients. Takata et al. observed ECG abnormalities more frequently in octogenarians with fewer teeth.[40] Their study did not find a significant relationship between ECG abnormalities and periodontitis and they believed that this was due to the advanced age of their subjects, who had lost many teeth. Joshipura et al. reported that men with few teeth who reported preexisting periodontal disease had an increased risk of CVD.[41] Indeed, in our study, the number of missing teeth was one of the significant risk indicators relevant to ECG abnormalities in the bivariate analysis, although the significance disappeared after the multivariate evaluation, which suggests that periodontitis, rather than the loss of many teeth, was the true risk factor for CVD. The lack of consistency between studies of the relationship between periodontitis and CVD is attributed to differences in the

age distribution of the subjects,[40,42] the method of evaluating periodontitis,[11] and study design.[13]

Our study found a relationship between periodontitis and ECG abnormalities, which suggests a relationship between periodontitis and CVD. Known risk factors for CVD, such as blood pressure, smoking status, alcohol consumption, and exercise frequency were also related to ECG abnormalities in the bivariate analyses; however, only periodontal condition, systolic blood pressure, and age were significant after the multivariate adjustment. The reported relative contribution of periodontitis to risk of CVD is generally small. In order to examine the degree of cardiovascular risk from periodontitis as compared to other risk factors, stringently designed cohort studies are required. Clinical intervention studies that examine whether treatment of periodontal disease reduces the risk of CVD will produce an answer.

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PAPER

Ten year recurrence after first ever stroke in a Japanese community: the Hisayama study

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Background: Very few population based cohort studies have focused on the long term recurrence of stroke.

Objective: To examine 10 year cumulative recurrence rates for stroke in a Japanese cohort according to pathological type and clinical subtype of brain infarction.

Methods: During a 32 year follow up of 1621 subjects ≥40 years of age, 410 developed first ever stroke. These were followed up prospectively for 10 years after stroke onset.

Results: During follow up, 108 (26%) experienced recurrent stroke. The cumulative recurrence rates were 35.3% at five years and 51.3% at 10 years. The 10 year recurrence rates of subarachnoid haemorrhage (SAH), brain haemorrhage, and brain infarction were 70.0%, 55.6%, and 49.7%, respectively; the difference between SAH and brain infarction was significant ($p=0.004$). Most recurrent episodes after SAH or brain haemorrhage happened within a year after the index stroke, whereas recurrence of brain infarction increased consistently throughout the observation period. Cardioembolic stroke had a higher recurrence rate (75.2%) than lacunar infarction (46.8%) ($p=0.049$). The 10 year risk of stroke recurrence increased with age after lacunar or atherothrombotic brain infarction, but not after the other types or subtypes. After atherothrombotic brain infarction, cardioembolic stroke, or SAH, the type and subtype of most recurrent strokes were the same as for the index stroke, but recurrence after lacunar infarction or brain haemorrhage showed divergent patterns.

Conclusions: Japanese people have higher recurrence rates of stroke than other populations. Recurrence rate after a first brain infarct increases consistently through the next 10 years.

Japanese people have high rates of morbidity and mortality from stroke.[1] Among stroke survivors, recurrence is common, resulting in cumulative disability and cognitive dysfunction.[2] Consequently, precise information is needed on the long term rates and determinants of recurrence after first stroke, so that clinical trials can be designed and health care policies for primary and secondary stroke prevention can be established. Most studies on stroke recurrence, reported mainly from Western countries, have been based on stroke registries[3–11] or on series of patients referred to hospitals.[12 13] A truly representative assessment of stroke recurrence in a community would require a prospective cohort of a defined population and an exhaustive follow up system. The Framingham study is the only cohort based examination of both initial and recurrent stroke, but it refers to the recurrence of thrombotic brain infarction only.[14] Stroke is divided into several pathological types. Among them, brain infarction is further classified into several clinical subtypes.[15–17] Very few studies, however, have accurately defined types and subtypes while also evaluating the long term risk of stroke recurrence.[3]

Since 1961, we have been carrying out a prospective cohort study of cardiovascular disease in the town of Hisayama, Japan.[18 19] The most outstanding features of this study are that the causes of death were verified by necropsy and that most of the stroke patients were examined morphologically at necropsy or, before death, by brain imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI). Our aim in this study was to estimate 10 year cumulative recurrence rates after first ever stroke in the community of Hisayama, using data stratified by sex, age, stroke type, and, in cases of brain infarction, the clinical subtype.

METHODS

Subjects and follow up surveys

In 1961, we carried out a screening examination among Hisayama residents and established a cohort consisting of 1621 stroke-free subjects aged ≥40 years (88.1% of the total population in this age range). These subjects were then followed up for 32 years, from 1 November 1961 to 31 October 1993. A detailed description of the study methods has been published previously.[18 19] In brief, we collected information about new cardiovascular events through a daily monitoring system established by the study team, local practitioners, and the town government. When we suspected a patient was having a new neurological symptom or a new deterioration of an already existing symptom, one of the physicians participating in the study would carefully evaluate the subject and try to obtain information by further diagnostic examinations, including lumbar puncture, cerebral angiography, or recent brain CT or MRI. During the 32 year period, all but two subjects were followed up and 1063 subjects died. Of those who died, 861 (81.0%) underwent necropsy.

The study was conducted with the approval of the human ethics review committee of Kyushu University Graduate School of Medical Sciences.

First ever stroke

Stroke, defined as the sudden onset of a non-convulsive and focal neurological deficit persisting for over 24 hours, was classified into four pathological types: brain infarction, brain haemorrhage, subarachnoid haemorrhage, and undetermined. Brain infarction was further divided into four clinical subtypes: lacunar infarction, atherothrombotic brain

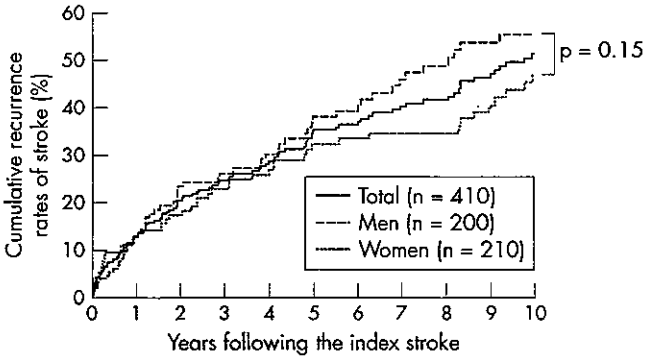

Figure 1 Kaplan–Meier estimates of cumulative recurrence rates of stroke for all subjects and for all subjects divided by sex. Deaths without stroke recurrence were censored.

infarction, cardioembolic stroke, and undetermined. These types and subtypes were defined on the basis of the *Classification of Cerebrovascular Disease III* proposed by the National Institute of Neurological Disorders and Stroke (USA).[15] The subtypes of ischaemic stroke were classified by TOAST (trial of Org 10172 in acute stroke treatment)[16] and by the Cerebral Embolism Task Force.[17] A detailed method of classifying stroke has been published previously.[19] The diagnosis and classification of stroke in our study were based on clinical history, neurological examination, all available clinical information (including brain CT or MRI), and necropsy findings.

During the 32 year follow up, we identified 410 first ever stroke events (200 men and 210 women, mean (SD) age, 73.9 (10.1) years), and divided them into 298 cases of brain infarction, 73 of brain haemorrhage, 35 of subarachnoid haemorrhage, and four undetermined. The cases of brain infarction by subtype consisted of 167 lacunar infarcts, 62 atherothrombotic brain infarcts, 56 cardioembolic strokes, and 13 undetermined.

Recurrent stroke

The definition of recurrent stroke was the same as that of index stroke, but with an additional criterion: there had to be either a new focal neurological deficit or a new deterioration of a previous deficit that was not attributed to brain oedema, haemorrhagic transformation after ischaemia, intercurrent illness, or iatrogenesis. This definition included recurrence in the early stage after the preceding stroke or recurrence in the same vascular territory as the preceding stroke.

We followed up the 410 patients with index stroke from the time of stroke onset until death or 31 August 2003. Under those conditions, all patients completed the follow up period. In the 10 years after the index stroke, 108 patients developed recurrent stroke. Of these, 88 had one recurrent stroke, 13 had two, six had three, and one had four. However, the end point of this study for each subject was the first recurrence.

Morphological evaluation

Brain imaging, including CT or MRI, was carried out in 153 (37%) of the 410 subjects with index stroke and in 43 (40%) of the 108 subjects with recurrent stroke. Necropsy findings were available in 332 (84%) of the 394 deceased stroke patients. As a result, morphological evaluation, including brain imaging or necropsy, was undertaken in 376 (92%) of the index stroke patients and 102 (94%) of the recurrent stroke patients until 31 August 2003.

Because we began collecting data on stroke subjects in 1961, imaging examinations of the brain and heart were non-existent in the early study period. However, we compensated

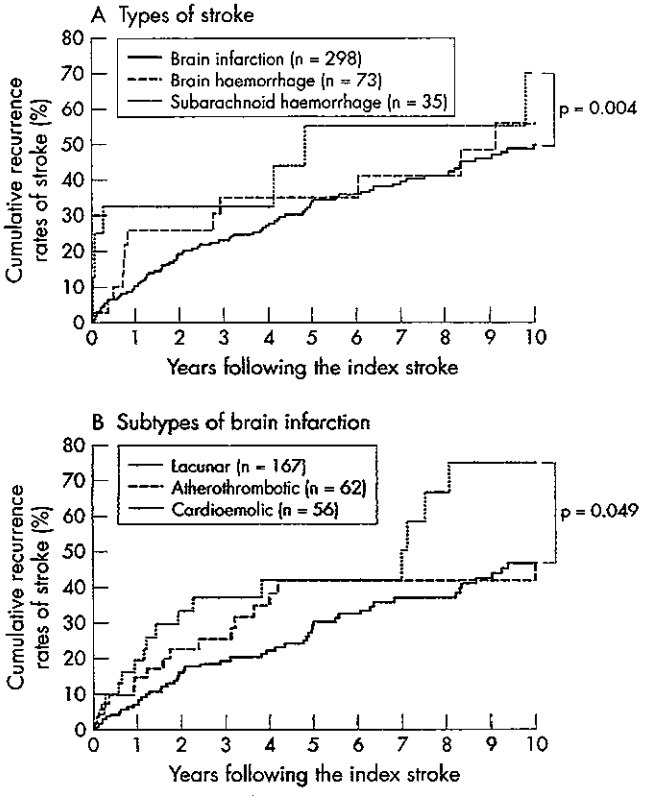

Figure 2 Kaplan–Meier estimates of cumulative recurrence rates of stroke according to stroke type (A) and, in cases of brain infarction, the subtype (B). Deaths without stroke recurrence were censored.

for this disadvantage by carrying out necropsy examinations on the vast majority of deceased patients. We reviewed the brains to evaluate the site, size, and pathological features of the stroke. We also investigated the heart and major vessels in detail—including the aorta, carotids, vertebrobasilar arteries, and the circle of Willis—in order to identify atherothrombotic stenotic lesions and embolic sources. In cases where the necropsy was carried out a long time after stroke onset, it was important to distinguish brain haemorrhage from brain infarction with haemorrhagic transformation. The latter was usually the result of a cardioembolic mechanism. When an infarcted area was surrounded by deposition of haemosiderin—with either no or mild atherosclerosis of the responsible artery, and given the presence of the embolic source—we considered the stroke lesion to be a brain infarct with haemorrhagic transformation. An old lesion that looked like a slit was considered to indicate a brain haemorrhage, especially if found in the basal ganglia or thalamus.

To classify the subtypes of brain infarction, we considered important the size and location of the infarcted area, the presence of stenosis or occlusion of a responsible cerebral artery, and the embolic source, in addition to clinical information including the disease course. Where multiple asymptomatic infarctions were present, we considered an infarct to be the lesion responsible for the stroke when it was most closely in accord with the neurological findings and disease course in the acute period of the stroke. The criteria for diagnosing brain infarction subtypes were given in full detail in our previous report.[19] When sufficient clinical and morphological information was obtained, a diagnosis of subtype was defined as "definite"; on the other hand, when either type of information was insufficient, the diagnostic level was defined as "probable." Among 298 cases of brain infarction, 272 were definite and 26 probable. In this study,

we present the data on the definite and probable cases together, as these combined data were almost identical to the data for definite cases only.

Statistical analysis

SAS software (version 6.12) was used for statistical analysis. The cumulative recurrence rates of stroke and the 95% confidence intervals (CI) were estimated by the Kaplan–Meier product limit method. The Cox proportional hazards model was used to test differences in recurrence rates as well as to estimate relative risks (RR) and 95% CIs of stroke recurrence.

RESULTS

Recurrence rates of stroke

Figure 1 shows the Kaplan–Meier estimates of cumulative recurrence rates of stroke for all subjects and for all subjects divided by sex. The recurrence rates (95% CI) at 1, 5, and 10 years were 12.8% (8.9% to 16.6%), 35.3% (29.0% to 41.5%), and 51.3% (43.8% to 58.9%), respectively, for all subjects. For men, these rates were 12.9% (7.3% to 18.5%), 38.1% (28.9% to 47.2%), and 55.6% (44.9% to 66.4%); for women the rates were 12.5% (7.3% to 17.6%), 32.3% (23.8% to 40.9%), and 47.1% (36.5% to 57.6%). The recurrence rates were slightly higher for men than for women, but the overall difference was not statistically significant ($p = 0.15$).

Figure 2, panel A, shows cumulative recurrence rates of stroke by type of index stroke. The recurrence rates at 1, 5, and 10 years were 10.0% (6.3% to 13.8%), 34.1% (27.3% to 40.9%), and 49.7% (41.4% to 57.9%) after brain infarction; 25.6% (9.0% to 42.2%), 34.9% (16.0% to 53.8%), and 55.6% (32.2% to 79.1%) after brain haemorrhage; and 32.5% (10.3% to 54.6%), 55.0% (25.6% to 84.4%), and 70.0% (39.0% to 100%) after subarachnoid haemorrhage, respectively. The 10 year recurrence rate of subarachnoid haemorrhage was significantly higher than that of brain infarction (RR = 2.89 (95% CI, 1.40 to 5.97); $p = 0.004$). Also, brain haemorrhage recurred at a slightly higher rate than brain infarction, but the difference was not statistically significant ($p = 0.52$). Annual recurrence rates after brain infarction were about 10% per year in the first two years and consistently about 4% per year afterward. On the other hand, 58.3% of recurrent episodes took place within a year after brain haemorrhage, and 66.7% within three months after subarachnoid haemorrhage.

Figure 2, panel B, shows the cumulative recurrence rates of stroke by clinical subtype of brain infarction. The recurrence rates at 1, 5, and 10 years were 7.2% (3.1% to 11.2%), 30.4% (22.1% to 38.7%), and 46.8% (36.6% to 56.9%) after lacunar infarction; 14.8% (4.5% to 25.0%), 42.0% (25.5% to 58.5%), and 46.9% (29.2% to 64.5%) after atherothrombotic brain infarction; and 19.6% (6.3% to 32.8%), 42.2% (23.8% to 60.6%), and 75.2% (52.6% to 97.8%) after cardioembolic stroke, respectively. Cardioembolic stroke had a significantly higher risk of 10 year recurrence than lacunar infarction (RR = 1.76 (95% CI, 1.00 to 3.11); $p = 0.049$). The recurrence rate of atherothrombotic brain infarction was slightly higher than that of lacunar infarction, but the difference was not statistically significant ($p = 0.59$).

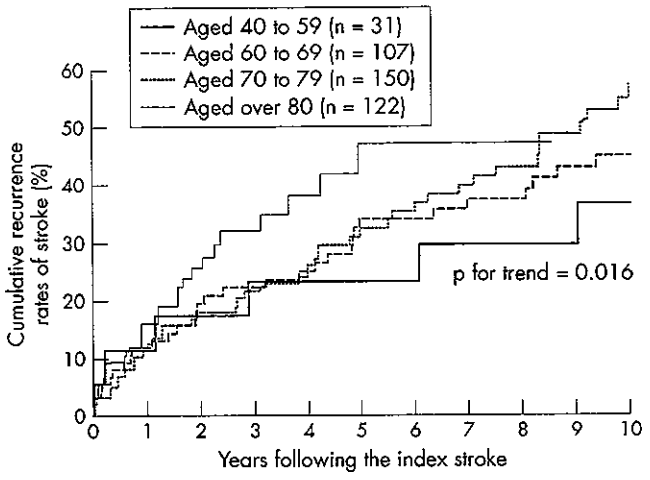

Figure 3 Kaplan–Meier estimates of cumulative recurrence rates of stroke for all subjects divided by age. Deaths without stroke recurrence were censored.

Figure 3 shows the cumulative recurrence rates of stroke by age. The 10 year risk of stroke recurrence was lowest in the youngest age group (40 to 59 years) and increased with age. Table 1 shows the relative risks of stroke recurrence among age groups during 10 years for each type and subtype of index stroke. The 10 year risk of stroke recurrence after brain infarction was lowest in the youngest age group and increased with age. For brain haemorrhage or subarachnoid haemorrhage, on the other hand, there was no significant relation between age and recurrence rates. Among the subtypes of brain infarction, the 10 year risk of recurrence after lacunar and atherothrombotic brain infarction was lowest in the youngest age group and increased with age, whereas for cardioembolic stroke there was no significant relation between age and recurrence rates.

Patterns of stroke recurrence

To evaluate patterns of stroke recurrence, table 2 shows the numbers and frequencies of first recurrent stroke by pathological types and clinical subtypes according to the type of index stroke. Most recurrent strokes after atherothrombotic brain infarction, cardioembolic stroke, or subarachnoid haemorrhage were the same type or subtype as the index stroke. On the other hand, recurrence after lacunar infarction or brain haemorrhage showed divergent patterns. The 51 patients who had recurrent stroke after lacunar infarction were divided as follows: 18 cases (35%) had a second lacunar infarction, 16 (31%) had atherothrombotic brain infarction, nine (18%) had brain haemorrhage, and six (12%) had cardioembolic stroke. Among the 12 recurrent cases of brain haemorrhage, seven (58%) had a second brain haemorrhage, three (25%) had lacunar infarction, and two (17%) had atherothrombotic or cardioembolic infarction.

DISCUSSION

One of the strengths of our study is that we investigated almost all stroke events occurring in a community based prospective cohort. Our study design eliminated the selection bias encountered in stroke registries or in series of hospital inpatients. Another strength is that recurrence rates were estimated up to 10 years after a subject's first ever stroke.

Recurrence rates of stroke

Three previous reports from stroke registries in Australia[3] and Britain[4 5] have reported five year cumulative stroke recurrence rates of 16.6% to 29.5%. In comparison, our study's five year cumulative stroke recurrence rate was 35.3%. There might be several reasons for this difference. First, there was a difference in methodology. The studies of the other three stroke registries all used a single set of criteria, which excluded vascular events occurring in the first 21 days after the index stroke unless such an event was clearly in a different vascular territory.[3–5] On the other hand, our study excluded neither early recurrence (10 cases within 21 days) nor recurrence in the same vascular territory. Second, race might greatly influence stroke recurrence. In our study,

Table 1 Relative risks and 95% confidence intervals of stroke recurrence during 10 years by age in each type or subtype of index stroke

	Age group (years)				
	40 to 59	60 to 69	70 to 79	80 and over	p Value
Index stroke	RR	RR (95% CI)	RR (95% CI)	RR (95% CI)	for trend
All types of stroke	1.0	1.3 (0.5 to 3.0)	1.6 (0.7 to 3.8)	2.2 (0.9 to 5.4)	0.016
Brain infarction	1.0	2.0 (0.6 to 6.5)	2.5 (0.7 to 8.1)	3.9 (1.1 to 13.1)	0.002
Lacunar infarction	1.0	2.2 (0.5 to 9.4)	2.6 (0.6 to 11.1)	4.8 (1.0 to 22.2)	0.022
Atherothrombotic brain infarction	1.0 *		1.8 (0.4 to 7.5)	4.7 (1.2 to 18.6)	0.001
Cardioembolic stroke	1.0	0.8 (0.1 to 7.3)	1.4 (0.2 to 12.3)	0.4 (0.0 to 4.1)	0.51
Brain haemorrhage	1.0	0.6 (0.0 to 6.3)	1.2 (0.2 to 10.3)	2.1 (0.2 to 24.3)	0.71
Subarachnoid haemorrhage	1.0	1.0 (0.2 to 6.0)	0.7 (0.1 to 4.4)	0.0	0.60

*Two age groups (40 to 59 and 60 to 69) were combined, as there were no recurrences after atherothrombotic brain infarction in the 40 to 59 age group.
CI, confidence interval; RR, relative risk.

haemorrhagic stroke—including brain haemorrhage and subarachnoid haemorrhage—recurred at higher rates than brain infarction, and the proportion of haemorrhagic stroke (26%) among all types was higher than those found in the three registries in Western countries (14% to 19%).[3–5] In addition, as Asians, including Japanese, have a higher stroke incidence than Europids,[1] they might also have higher rates of stroke recurrence.

In our study, most recurrent episodes occurred within a year after the index haemorrhagic stroke. This may indicate the importance of controlling risk factors and of treating the patient to prevent recurrence without delay in the first days and months after the onset of haemorrhagic stroke. On the other hand, cumulative recurrence rates after brain infarction, especially lacunar infarction, increased steadily during our 10 year study period. The Oxfordshire Community Stroke Project[6] also showed that the recurrence rate after lacunar infarction was low and almost constant throughout the follow up period. Arteriosclerosis, which is thought to progress consistently for a long period, may be related to recurrent thrombotic infarction. Thus careful observation and adequate treatment to prevent recurrence are needed for a long time after brain infarction.

Several studies have focused on the relations between brain infarction subtypes and the risks of recurrent stroke,[3 7–10 12] but their findings are equivocal. Some of those studies have claimed that the subtype of brain infarction is not a predictor of long term recurrence,[3 7 8] while others showed that the highest risk of recurrence is with atherothrombotic brain infarction.[9 10 12] In our study, cardioembolic stroke had the highest risk of recurrence among the three major subtypes of brain infarction. This is probably attributable to our inclusion of early recurrent episodes, which were often observed after cardioembolic stroke.[20 21]

In some studies,[3 11] aging was found to be a predictor of stroke recurrence. In the present study, the risk of recurrence after first ever lacunar or atherothrombotic brain infarction was lowest in the youngest age group and then increased with age. Aging would accelerate atherosclerotic changes in major cerebral arteries and arteriolosclerotic changes in penetrating arteries, thus increasing the risk of recurrent stroke.

Patterns of stroke recurrence

In the present study, the types or subtypes of most recurrent strokes after atherothrombotic brain infarction, cardioembolic stroke, or subarachnoid haemorrhage were the same as those of the index stroke. On the other hand, recurrence after lacunar infarction or brain haemorrhage showed divergent patterns. This finding was also emphasised in some previous reports.[4 13]

Several aetiological mechanisms for lacunar infarction have been proposed[22–24]: lipohyalinosis or microatheroma in a penetrating artery; branch-atheromatous disease, which is located in basilar or middle cerebral arteries and occludes the origins of one or more penetrating arteries; and microembolism from carotid or cardiac disease. These multifactorial aetiologies would support divergence in the type and subtype of recurrent stroke after lacunar infarction. Our findings denote the importance of evaluation to detect any large vessel disease or embolic source, even in patients with lacunar infarction.

Table 2 The numbers and frequencies of first recurrent stroke by pathological types and clinical subtypes according to type of index stroke

	Type or subtype of recurrent stroke								
		Subtype of BI							
Type or subtype of index stroke	All BI	LA	AT	CE	UND-BI	BH	SAH	UND	Total
Brain infarction	74 (85%)					10 (11%)	–	3 (3%)	87 (100%)
Lacunar infarction		18 (35%)	16 (31%)	6 (12%)	–	9 (18%)	–	2 (4%)	51 (100%)
Atherothrombotic brain infarction		1 (6%)	14 (82%)	–	1 (6%)	1 (6%)	–	–	17 (100%)
Cardioembolic stroke		–	–	16 (94%)	1 (6%)	–	–	–	17 (100%)
Undetermined subtype of BI (UND-BI)		–	–	–	1 (50%)	–	–	1 (50%)	2 (100%)
Brain haemorrhage	5	3 (25%)	1 (8%)	1 (8%)	–	7 (58%)	–	–	12 (100%)
Subarachnoid haemorrhage	2	1 (11%)	1 (11%)	–	–	1 (11%)	6 (67%)	–	9 (100%)
Undetermined type of stroke	–	–	–	–	–	–	–	–	0 (0%)

Percentages are the proportions of types or subtypes of recurrent stroke calculated using the numbers of total recurrent stroke as the denominators.
AT, atherothrombotic brain infarction; BH, brain haemorrhage; BI, brain infarction; CE, cardioembolic stroke; LA, lacunar infarction; SAH, subarachnoid haemorrhage; UND, undetermined.

Hypertension is a major risk factor for both lacunar infarction and brain haemorrhage, and lesions of all lacunar infarcts and most brain haemorrhages in our patients were located in brain areas that have the common feature of penetrating arteries, such as the basal ganglia, thalamus, and pons. These similarities would support the overlap between lacunar infarction and brain haemorrhage in recurrent stroke types.

Study limitations

There are several potential limitations to the findings in our study. First, we enrolled stroke cases that developed among an inception cohort during 32 years of follow up. The prevalence of cardiovascular risk factors and the risk of stroke recurrence may have changed widely during this long term observation period.[25] Secular trends in stroke recurrence should be examined, and we will do so in another study. Second, the study did not consider the effects of cardiovascular risk factors or those of medical or surgical treatment. Thus our estimates for the risk of stroke recurrence are probably quite conservative. Third, brain imaging was available in only 37% of the index stroke cases. However, we collected available clinical information on both index and recurrent strokes in minute detail and carried out necropsies on 84% of deceased stroke patients. We believe that our exhaustive and careful evaluation of the clinical information, as well as the high rate of necropsy, improved the quality and validity of the diagnosis as well as the stroke classification in our study.

Conclusions

Our findings show higher recurrence rates of stroke in a Japanese community than in Western populations. The divergent patterns of stroke recurrence after index lacunar infarction or brain haemorrhage are of interest and importance for the prevention of recurrent stroke, because the Japanese are characterised by high morbidity of lacunar infarction and brain haemorrhage. The consistent increase in cumulative recurrence rates during the long observation period and the higher recurrence rates after index brain infarction among older patients are both important for medical care. We believe that these findings will contribute to a better understanding of stroke recurrence in the Japanese, who are considered to be at greater risk of stroke than other populations.

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Competing interests: none declared

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Futile short-patch DNA base excision repair of adenine:8-oxoguanine mispair

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ABSTRACT

8-Oxo-7, 8-dihydrodeoxyguanosine (8-oxo-dG), one of the representative oxidative DNA lesions, frequently mispairs with the incoming dAMP during mammalian DNA replication. Mispaired dA is removed by post-replicative base excision repair (BER) initiated by adenine DNA glycosylase, MYH, creating an apurinic (AP) site. The subsequent mechanism ensuring a dC:8-oxo-dG pair, a substrate for 8-oxoguanine DNA glycosylase (OGG1), remains to be elucidated. At the nucleotide insertion step, none of the mammalian DNA polymerases examined exclusively inserted dC opposite 8-oxo-dG that was located in a gap. AP endonuclease 1, which possesses 3′→5′ exonuclease activity and potentially serves as a proofreader, did not discriminate dA from dC that was located opposite 8-oxo-dG. However, human DNA ligases I and III joined 3′-dA terminus much more efficiently than 3′-dC terminus when paired to 8-oxo-dG. In reconstituted short-patch BER, repair products contained only dA opposite 8-oxo-dG. These results indicate that human DNA ligases discriminate dC from dA and that MYH-initiated short-patch BER is futile and hence this BER must proceed to long-patch repair, even if it is initiated as short-patch repair, through strand displacement synthesis from the ligation-resistant dC terminus to generate the OGG1 substrate, dC:8-oxo-dG pair.

INTRODUCTION

The integrity of genomic DNA is maintained by accurate DNA replication and elaborate DNA repair in living cells. Among various threats to genetic information, oxidative damage to DNA is most abundant and inevitable as cells produce reactive oxygen species through energy metabolism (1). One of the oxidative DNA lesions, 8-oxo-7, 8-dihydrodeoxyguanosine (8-oxo-dG), is deleterious as it frequently mispairs with the incoming dAMP during DNA replication (2), leading to G:C→T:A transversions (3,4). Adenine paired to 8-oxo-dG is recognized and removed by adenine DNA glycosylase, MYH (MUTYH), a mammalian homolog of bacterial MutY (5,6). Recently, it was reported that inherited defects in the human *MYH* gene were associated with multiple colorectal tumors and somatic G→T mutations in the adenomatous polyposis coli (*APC*) gene (7). Furthermore, knockout of the mouse *MYH* gene resulted in spontaneous cancer (8) and a mutator phenotype in embryonic stem cells (9). These pieces of evidence emphasize the importance of the MYH-initiated base excision repair (BER) in cancer/mutation avoidance.

MYH is an adenine DNA glycosylase and initiates post-replication BER by removing adenine residues from DNA when paired to 8-oxo-dG or dG (5,6). Apurinic (AP) sites generated by MYH glycosylase is cleaved by AP endonuclease (APE), generating 3′-OH and 5′-deoxyribose phosphate (dRP). The 3′-OH residue serves as a primer terminus for a repair synthesis. When short-patch BER proceeds, 1 nt gap is converted to a nick by the actions of gap-filling DNA polymerase (pol) and deoxyribophosphodiesterase (dRPase). When long-patch BER proceeds, a strand displacement synthesis and the excision of a displaced strand by flap-endonuclease 1 (FEN1, DNase IV) are required to produce a ligatable nick (6,10). In both the cases, ligation of the nick follows to complete repair reactions. In BER of 8-oxo-dG:dA to 8-oxo-dG:dC, the mechanism following the removal of adenine base is not understood well. Unlike regular BER, the DNA glycosylase, MYH, removes the undamaged base, adenine, and DNA polymerase inserts a nucleotide opposite the lesion, 8-oxo-dG. The reactions must ensure the formation of 8-oxo-dG:dC pair, which is then repaired to dG:dC by regular BER initiated by 8-oxoguanine DNA glycosylase (OGG1). MYH is located in replication foci (11) and interacts with the proliferating cell nuclear antigen (PCNA), APE1, MSH6 and RPA (12,13). The post-replication repair is coupled with replication (14) and is suggested to follow long-patch BER (12,15,16). However, even if the long-patch BER is the mechanism for the repair, the reason for this choice is not clear. It is plausible that 8-oxo-dG inhibits ligation of a nucleotide inserted opposite this lesion during short-patch BER, hence the repair proceeds to the long-patch repair pathway.

To gain insights into the post-replication repair, we examined the three steps (nucleotide insertion, proofreading and ligation) that are involved in the repair. We also reconstituted short-patch BER of 8-oxo-dG:dA with purified proteins.

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MATERIALS AND METHODS

Enzymes

Mouse MYH was purified as described previously (9). Human APE1, PCNA and calf thymus DNA polymerase δ were provided by Carlos de los Santos, Paul Fisher and Holly Miller (SUNY at Stony Brook), respectively. Human DNA polymerases, pol λ, pol η, pol κ, pol β and pol ι, were provided by Luis Blanco (Universidad Autonoma), Fumio Hanaoka (Osaka University), Haruo Ohmori (Kyoto University), Holly Miller (SUNY) and Roger Woodgate (NIH), respectively. Human DNA ligases I and IIIβ were provided by Alan Tomkinson (University of Maryland Medicine).

Expression and purification of human X-ray repair cross complementing 1 (XRCC1) and DNA ligase IIIα

Plasmids expressing histidine-tagged human XRCC1 (17) and DNA ligase IIIα (18) were provided by Larry Thompson (Lawrence Livermore National Laboratory) and Tomas Lindahl (Cancer Research UK), respectively. *Escherichia coli* BL21-CodonPlus(DE3)-RIL (Stratagene) was transformed and the protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside at 1 mM. After 2 h incubation at 37°C, bacteria were harvested and suspended in a buffer consisting of 50 mM Tris–HCl (pH 7.5), 10% glycerol, 0.5 M NaCl, 5 mM 2-mercaptoethanol and 1 mM imidazole. Bacteria were sonicated and a crude extract was obtained by ultracentrifugation. The extract was applied to a Ni-NTA agarose column (QIAGEN), and the column was washed successively with 1, 40 and 80 mM imidazole-containing buffers. XRCC1 and DNA ligase IIIα proteins were eluted with a 250 mM imidazole-containing buffer. Identity and purity of proteins were confirmed by SDS–PAGE. Proteins were dialyzed against a buffer containing 50 mM Tris–HCl (pH 7.5), 50% glycerol, 0.1 M NaCl, 1 mM EDTA and 10 mM 2-mercaptoethanol and then stored at −20°C. Protein concentration was determined with Protein Assay reagent (BioRad) using BSA as a standard.

Oligonucleotides

All oligonucleotides were synthesized by the DNA synthesis facility (SUNY, Stony Brook) and purified by electrophoresis in a denaturing 20% polyacrylamide gel. 5′-End labeling was carried out using [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Biosciences) and T4 polynucleotide kinase (New England BioLabs). Labeled oligonucleotides were purified by a Micro-Spin G-25 column (Amersham Biosciences). For the preparation of duplex DNA, oligonucleotides were incubated at 80°C for 10 min in a solution (50 μl) containing 0.1× TE and 100 mM NaCl and then slowly cooled to room temperature.

Template oligonucleotides (40mer) were 5′CCAACTTG-AAAACGCTCCACXATACCTTACATGCTAGAAC where X = dG or 8-oxo-dG. For nucleotide insertion experiments, 5′-^{32}P-labeled 19mer primer (5′GTTCTAGCATGTAAG-GTAT) and 5′-phosphorylated downstream 20mer oligonucleotide (5′GTGGAGCGTTTTCAAGTTGG) were annealed to template 40mer, forming a 1 nt gap in the middle of duplex DNA. For DNA ligation and exonuclease assays, 5′-^{32}P-labeled 20mer oligonucleotides (5′GTTCTAGCATGTAAG-GTATX, where X = dA, dC, dG or dT) and 5′-phosphorylated downstream 20mer oligonucleotide (described above) were annealed to template 40mer, generating a substrate with a correctly paired or mispaired 3′ terminus at a nick in the middle of duplex DNA. For MYH-initiated reconstitution experiments, dA:8-oxo-dG mispair-containing 40mer/40mer duplex DNA was employed.

Nucleotide insertion reaction

The reaction mixture (20 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 30 mM KCl, 5 mM DTT, 100 μg/ml BSA, 25 nM 5′-labeled primer/template, 2.5 nM enzyme (pol β, pol η, pol ι, pol κ or pol λ) and dATP or dCTP (concentrations are indicated in Figure 1). For reactions with pol δ, the mixture (20 μl) contained 40 mM bis-Tris (pH 6.7), 6 mM $MgCl_2$, 10 mM DTT, 250 μg/ml BSA, 25 nM 5′-labeled primer/template, 110 nM PCNA (as a trimer), 2.25 U pol δ and dATP or dCTP. For reaction containing both dATP and dCTP, the mixture (20 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 30 mM KCl, 10 mM DTT, 250 μg/ml BSA, 25 nM unlabeled primer/template, 2.5 nM enzyme (pol β, pol η or pol λ), 0.5 μM dATP, 0.5 μM dCTP and 6.6 nM (3000 Ci/mmol) of [α-^{32}P]dATP or [α-^{32}P]dCTP. The reaction was conducted at 37°C for 10 min and terminated by adding 20 μl of a formamide loading buffer (96% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 19 mM EDTA) followed by heating at 90°C for 3 min. The products were analyzed by electrophoresis in a denaturing 20% polyacrylamide gel. Radioactive DNA bands were visualized and quantified by a Storm840 PhosphorImager and ImageQuant software (Amersham Biosciences).

Assay for 3′→5′ exonuclease activity of APE1

The reaction mixture (20 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 30 mM KCl, 5 mM DTT, 50 μg/ml BSA, 10 nM 5′-labeled substrate and 0, 1 or 5 nM APE1. The reaction was performed at 37°C for 30 min and terminated as described above.

DNA ligation reaction

The reaction mixture (20 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 1 mM ATP, 50 μg/ml BSA, 100 mM KCl, 10 nM 5′-labeled substrate and human DNA ligases I (0, 5 and 25 nM), IIIα (0, 0.5 and 2.5 nM) or IIIβ (0, 5 and 25 nM). The ligation mixture was incubated at 37°C for 30 min, after which products were analyzed by gel electrophoresis as described above. In time-course experiments (Figure 4), 50 nM DNA ligase I or 10 nM DNA ligase IIIα/XRCC1 was used in 200 μl of the reaction mixture containing 2 mM ATP (instead of 1 mM) and 0 or 150 mM KCl. Aliquots (10 μl) were removed at various time points during incubation at 37°C, quenched and analyzed for products as described above.

Adenine base excision catalyzed by MYH and APE1

The reaction mixture (20 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM DTT, 50 μg/ml BSA, 1 nM 5′-labeled 40mer duplex DNA and MYH (0, 2.5, 5 or 10 nM) and was incubated at 37°C for 15 min followed by incubation with APE1 (0, 10 or 50 nM) in the presence of 150 mM KCl at 37°C for 15 min (Figure 5).

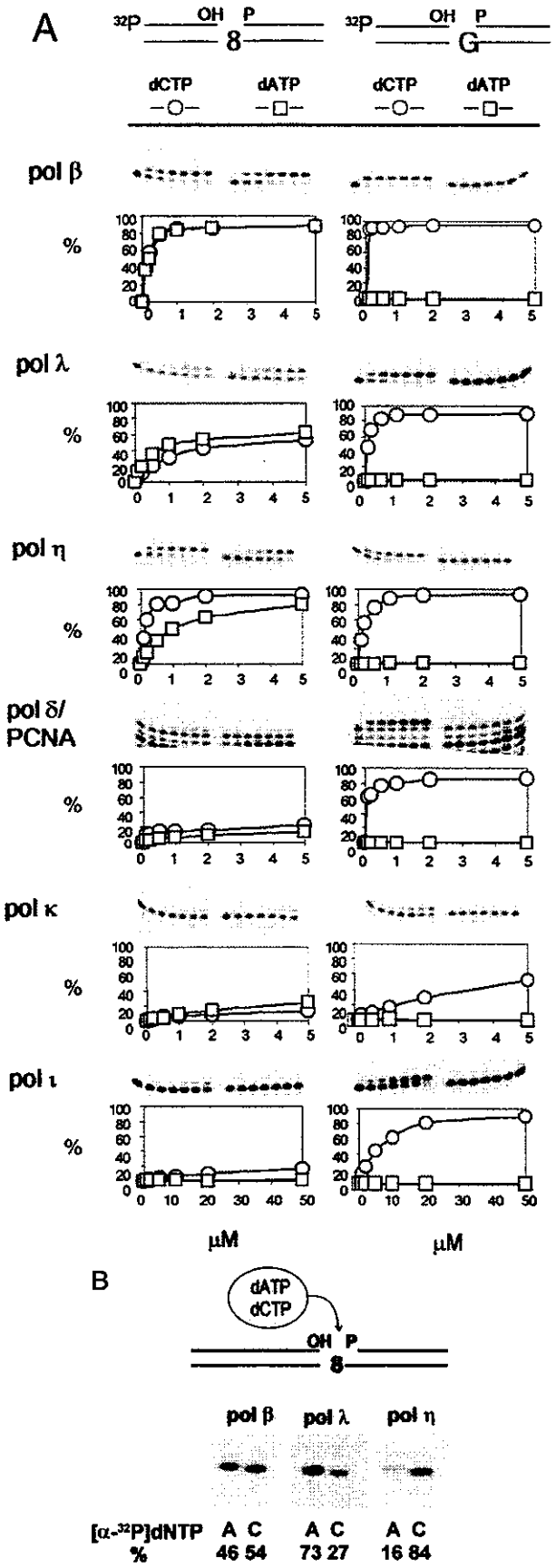

Figure 1. Nucleotide insertion opposite 8-oxo-dG. (**A**) Insertion of dAMP or dCMP opposite 8-oxo-dG was determined using 40mer duplex DNA that had a 1 nt gap opposite the lesion. Substrate DNA is shown on the top. Here, '8' stands for 8-oxo-dG. Intensities of radioactive bands were measured and insertion frequency, represented as percent, was plotted as a function of nucleotide concentration (μM). (**B**) Nucleotide insertion was determined in the presence of both 0.5 μM dATP and 0.5 μM dCTP. Either [α-^{32}P]dATP or [α-^{32}P]dCTP was included in the reaction mixture as a tracer. Relative ratio of insertions of dAMP and dCMP was shown in percentage.

Reconstitution of MYH-initiated short-patch BER

A starting reaction mixture (100 μl) contained 1 nM unlabeled 40mer duplex DNA and 10 nM MYH and was incubated at 37°C for 30 min. Following the reaction, the mixture was supplemented with 150 mM KCl, 2 mM ATP, 0.5 μM dATP, 0.5 μM dCTP and 33 nM of [α-^{32}P]dATP or [α-^{32}P]dCTP (6000 Ci/mmol), 100 nM APE1, 4 nM pol β and 10 nM of DNA ligase IIIα/XRCC1, and then incubated at 37°C. Aliquots (10 μl) were removed at various time points and the products were analyzed as described above.

RESULTS

Both dAMP and dCMP are inserted opposite 8-oxo-dG by various DNA polymerases

The consecutive action of MYH and APE1 produces a 1 nt gap opposite 8-oxo-dG with 3′-OH group and 5′-dRP moiety, and 8-oxo-dG serves as a template for the repair synthesis. We examined the insertion specificity of several DNA polymerases using a 1 nt gapped substrate (Figure 1A and B). Among DNA polymerases examined, pol β, pol η and pol λ inserted a nucleotide opposite 8-oxo-dG more efficiently than do pol κ, pol ι and pol δ/PCNA. Regarding their specificities, pol η and its close relative, pol ι, preferred dC to dA; pol β and pol δ/PCNA appeared to insert dC and dA at similar frequencies; and pol λ and pol κ preferred dA to dC. All these pols inserted dC opposite dG (Figure 1A) though the activities of pol κ and pol ι were weak when a gapped substrate was used. These results indicate that none of the DNA polymerases exclusively inserts dC opposite 8-oxo-dG.

APE1 exonuclease activity does not discriminate dA from dC pairing to 8-oxo-dG

APE1 is reported to have a 3′→5′ exonuclease activity active on 3′-mismatched termini of nicked and gapped DNA molecules and is suggested to compensate for the lack of a proofreading function of pol β (19). This finding raises the possibility that APE1 removes selectively 3′-dA, but not dC, inserted opposite 8-oxo-dG during a gap-filling reaction. To explore this possibility, we used four substrates with different termini at nicks (Figure 2). When dG was a template, all mismatched termini, but not 3′-dC, were subjected to proofreading. When 8-oxo-dG was a template, all four termini were proofread: there was no difference between 3′-dC and 3′-dA, and this result was true under three different KCl concentrations (0, 30 and 150 mM). The exonuclease activity of APE1 was progressively impaired by KCl. We conclude that APE1 does not discriminate 3′-dA from 3′-dC inserted opposite 8-oxo-dG.

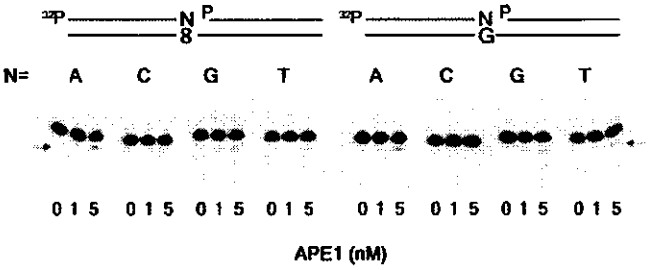

Figure 2. 3′→5′ Exonucleolytic proofreading activity of APE1. Nicked DNA substrate is shown above the panel. 'N' stands for dA, dC, dG or dT. Arrows indicate proofread products.

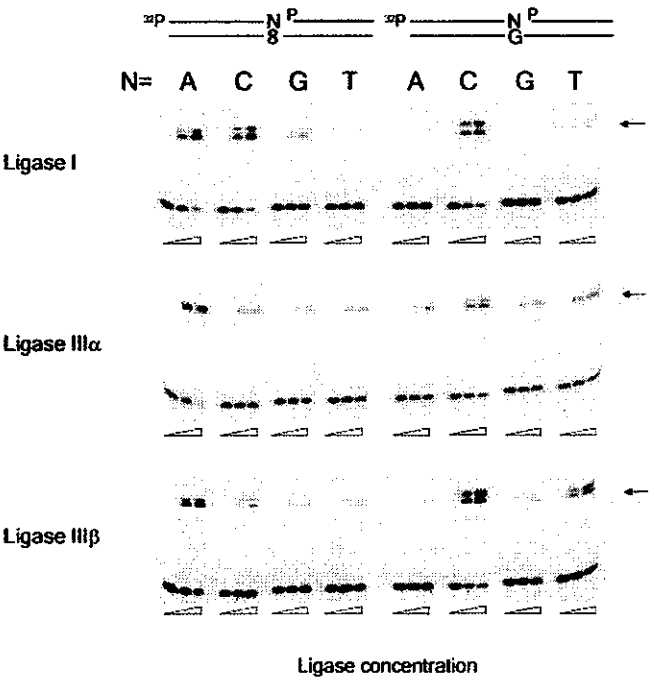

Figure 3. Ligation of four 3′-termini pairing with 8-oxo-dG or dG to downstream DNA. Nicked DNA substrate is shown on the top. Ligation products are 40mer indicated by arrows. All oligonucleotides used in this study were purified by gel electrophoresis and showed one band, but ligation products always appeared as two bands. We do not know the reason for this. The concentrations of ligases were 0, 0.5 and 2.5 nM for ligase IIIα and 0, 5 and 25 nM for ligase I and IIIβ.

DNA ligases ligate 3′-dA terminus much more efficiently than 3′-dC terminus when pairing to 8-oxo-dG

If a DNA ligase is active on only 3′-dC terminus pairing to 8-oxo-dG, the ligation step will play a critical role in the short-patch BER of dA:8-oxo-dG mispair. We compared the degrees of ligation of four 3′-termini pairing to 8-oxo-dG. Human DNA ligase I, which is believed to be engaged in DNA replication, long-patch BER and nucleotide excision repair appeared to ligate both 3′-dA and 3′-dC termini at similar efficiencies in a qualitative experiment (Figure 3). The other two termini, dG and dT, were poorly ligated by this enzyme. DNA ligases IIIα and IIIβ, which are produced by an alternative splicing (20), appeared to be much more active on 3′-dA than on the other termini. When unmodified DNA

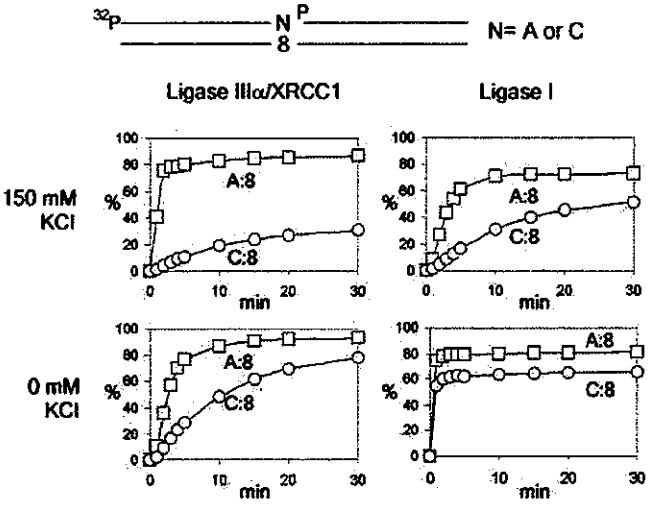

Figure 4. Time course of DNA ligation catalyzed by human DNA ligase I and ligase III/XRCC1 in the presence (150 mM) or in the absence of KCl. Substrate DNA was the same as in Figure 3, and 'N' stands for A or C. The *x*-axis and *y*-axis represent incubation time (min) and generation of ligation products (percent), respectively.

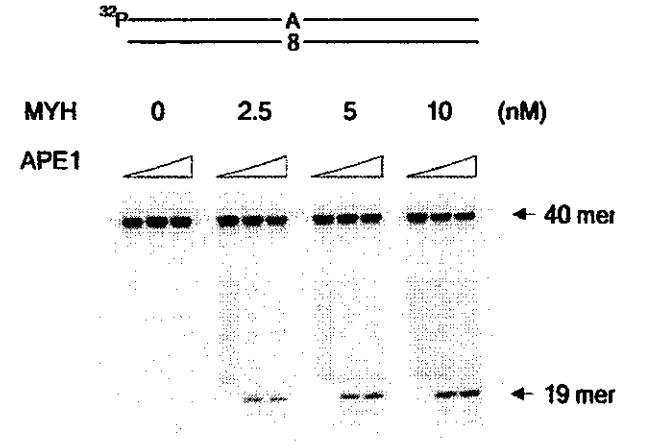

Figure 5. Adenine base excision catalyzed by MYH and APE1. 5′-Labeled duplex 40mer containing dA:8-oxo-dG pair was incubated with MYH at 37°C for 15 min followed by 15 min incubation with APE1 (0, 10 or 50 nM) in the presence of 150 mM KCl.

was used as a substrate, ligase I exclusively ligated a 3′-dC terminus pairing to dG, whereas ligases IIIα and IIIβ ligated a dT terminus as well as a dC terminus. Since DNA ligase IIIα is believed to be the ligase involved in short-patch BER, we further characterized ligation catalyzed by this enzyme of 3′-dA and 3′-dC termini pairing to 8-oxo-dG (Figure 4). Burst DNA joining occurred on the dA terminus, but not on the dC terminus, in a reaction mixture containing 150 mM KCl, which is considered to represent the physiological salt condition (21). Ligation of the dA terminus was also more efficient than that of the dC terminus in the absence of KCl, but the difference was less drastic. The effects of varying amounts of XRCC1 on the ligation of 3′-dA and -dC termini were also