Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis

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Lancet 2007; 369: 1090–98

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Summary

Background Epidemiological and clinical evidence suggests that an increased intake of long-chain n-3 fatty acids protects against mortality from coronary artery disease. We aimed to test the hypothesis that long-term use of eicosapentaenoic acid (EPA) is effective for prevention of major coronary events in hypercholesterolaemic patients in Japan who consume a large amount of fish.

Methods 18 645 patients with a total cholesterol of 6·5 mmol/L or greater were recruited from local physicians throughout Japan between 1996 and 1999. Patients were randomly assigned to receive either 1800 mg of EPA daily with statin (EPA group; n=9326) or statin only (controls; n=9319) with a 5-year follow-up. The primary endpoint was any major coronary event, including sudden cardiac death, fatal and non-fatal myocardial infarction, and other non-fatal events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass grafting. Analysis was by intention-to-treat. The study was registered at clinicaltrials.gov, number NCT00231738.

Findings At mean follow-up of 4·6 years, we detected the primary endpoint in 262 (2·8%) patients in the EPA group and 324 (3·5%) in controls—a 19% relative reduction in major coronary events (p=0·011). Post-treatment LDL cholesterol concentrations decreased 25%, from 4·7 mmol/L in both groups. Serum LDL cholesterol was not a significant factor in a reduction of risk for major coronary events. Unstable angina and non-fatal coronary events were also significantly reduced in the EPA group. Sudden cardiac death and coronary death did not differ between groups. In patients with a history of coronary artery disease who were given EPA treatment, major coronary events were reduced by 19% (secondary prevention subgroup: 158 [8·7%] in the EPA group *vs* 197 [10·7%] in the control group; p=0·048). In patients with no history of coronary artery disease, EPA treatment reduced major coronary events by 18%, but this finding was not significant (104 [1·4%] in the EPA group *vs* 127 [1·7%] in the control group; p=0·132).

Interpretation EPA is a promising treatment for prevention of major coronary events, and especially non-fatal coronary events, in Japanese hypercholesterolaemic patients.

Introduction

Epidemiological and clinical evidence suggests a significant inverse association between long-term intake of long-chain n-3 polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and mortality associated with coronary artery disease.[1–7] Thus, the consumption of fish or fish-oil could protect against major events associated with coronary artery disease, especially fatal myocardial infarction and sudden cardiac death. Two large-scale secondary prevention trials, the Diet and Reinfarction Trial and the Gruppo Italiano per lo Studio della Sopravivenza nell' Infarto Miocardico-Prevenzione Trial, reported that increased consumption of fish or fish-oil supplements reduced coronary death in postinfarction patients.[8,9] No randomised trials have examined the effects of n-3 polyunsaturated fatty acids on major coronary events in a high-risk, primary prevention population.

EPA ethyl ester, which is purified from n-3 polyunsaturated fatty acids present in fish oil, is approved by Japan's Ministry of Health, Labour, and Welfare as a treatment for hyperlipidaemia and peripheral artery disease. The biological functions of EPA include reduction of platelet aggregation,[10,11] vasodilation,[12,13] antiproliferation,[14] plaque-stabilisation,[15] and reduction in lipid action.[16,17] Therefore the preventive effects of EPA on major cardiovascular events are of both clinical interest and therapeutic importance.

Primary and secondary prevention trials have proved that cholesterol-lowering treatment with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase—statins—reduces the risk of all-cause mortality and major cardiovascular events in patients with a wide range of cholesterol concentrations, whether or not they have had coronary artery disease.[18–21] Thus, statins are now established as the first-line treatment for hyperlipidaemia.[22] Preliminary data for treatment with a combination of n-3 polyunsaturated fatty acids and statins have shown beneficial effects on the lipid profiles of patients with a mixed type of hyperlipidaemia;[23–25] however, no major long-term inter-

ventional trial has yet investigated whether the addition of EPA to conventional statin treatment would yield an incremental clinical benefit. The Japan EPA Lipid Intervention Study (JELIS) tests the hypothesis that long-term use of EPA is effective in reduction of major coronary events in Japanese hypercholesterolaemic patients given statins.

Methods

Study design and patients

We did a prospective, randomised open-label, blinded endpoint evaluation (PROBE).[26] Our study design, and inclusion and exclusion criteria are described in detail elsewhere.[27] We recruited 19466 hypercholesterolaemic patients through local physicians from all regions of Japan between November, 1996, and November, 1999. Figure 1 shows the trial profile. The participants consisted of 5859 men (aged 40–75 years) and 12786 postmenopausal women (aged up to 75 years), with or without coronary artery disease, which was defined as previous myocardial infarction, coronary interventions, or confirmed angina pectoris. Informed written consent was obtained from all eligible patients before random assignment to either the EPA treatment or control groups.

Eligibility criteria were total cholesterol concentration of 6·5 mmol/L or greater, which corresponded to a LDL cholesterol of 4·4 mmol/L or greater. Exclusion criteria were: acute myocardial infarction within the past 6 months, unstable angina pectoris, a history or complication of serious heart disease (such as severe arrhythmia, heart failure, cardiomyopathy, valvular disease, or congenital disease), cardiovascular reconstruction within the past 6 months, cerebrovascular disorders within the past 6 months, complications of serious hepatic or renal disease, malignant disease, uncontrollable diabetes, hyperlipidaemia due to other disorders, hyperlipidaemia caused by drugs such as steroid hormones, haemorrhage (including haemophilia, capillary fragility, gastrointestinal ulcer, urinary tract haemorrhage, haemoptysis, and vitreous haemorrhage), haemorrhagic diathesis, hypersensitivity to the study drug formulation, patients' intention to undergo surgery, and judgment by the physician in charge that a patient was inappropriate for the study.

The primary endpoint was any major coronary event, including sudden cardiac death, fatal and non-fatal myocardial infarction, and other non-fatal events including unstable angina pectoris, angioplasty, stenting, or coronary artery bypass grafting. Secondary endpoints (all-cause mortality, mortality and morbidity of coronary artery disease, stroke, peripheral artery disease, and cancer) are not reported here.

Procedures

We used the statistical coordination centre at the Toyama Medical and Pharmaceutical University to manage patient registration (including confirmation of eligibility criteria), operation of the randomisation scheme, and data management. We used permuted-block randomisation with a block size of four. Blocks were assigned according to the number of participants enrolled at each centre. Patients were divided into two subgroups: one with coronary artery disease (secondary prevention; n=3664) and one without (primary prevention; n=14981), and stratified accordingly. Patients were randomly assigned to receive EPA with statin (EPA group) or statin alone (controls). All patients first underwent 4–8 weeks of washout from antihyperlipidaemic drugs. Patients also received appropriate dietary advice.

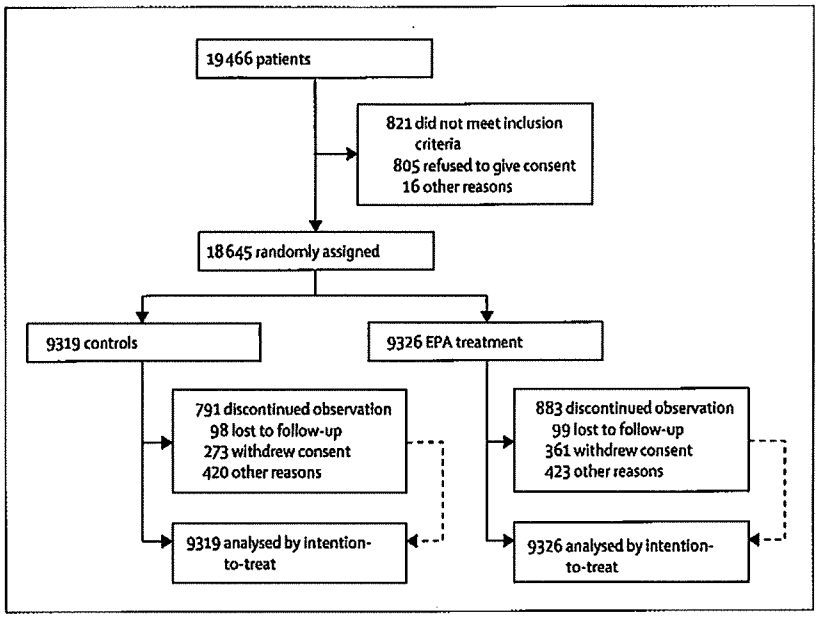

Figure 1: **Trial profile**

All patients received 10 mg of pravastatin or 5 mg of simvastatin once daily as first-line treatment. These statins were available in Japan at the initiation of this study, and these doses were recommended by the Ministry of Health, Labour, and Welfare. For serious hypercholesterolaemia (defined as uncontrolled), this daily dose was increased to 20 mg pravastatin or 10 mg simvastatin. No treatment with other antihyperlipidaemic drugs was allowed during the study. EPA was given at a dose of 600 mg, three times a day after meals (to a total of 1800 mg per day). We used capsules that contained 300 mg of highly purified (>98%) EPA ethyl ester (Mochida Pharmaceuticals, Tokyo, Japan).

Local physicians monitored compliance with dietary advice and medication, and noted adverse events at every clinic visit. Clinical endpoints and severe adverse events reported by local physicians were checked by members of a regional organising committee in a blinded fashion. Then, an endpoints adjudication committee (see webappendix), consisting of three expert cardiologists and one expert neurologist, confirmed them once a year without knowledge of the

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	Controls (n=9319)	EPA treatment (n=9326)
Age (years)	61 (9)	61 (8)
Male	2908 (31%)	2951 (32%)
BMI (kg/m²)	24 (3)	24 (3)
Cardiovascular history		
Myocardial infarction	502 (5%)	548 (6%)
Angina	1484 (16%)	1419 (15%)
CABG or PTCA	433 (5%)	462 (5%)
Risk factors		
Smoking	1700 (18%)	1830 (20%)
Diabetes	1524 (16%)	1516 (16%)
Hypertension	3282 (35%)	3329 (36%)
Serum lipid values		
Total cholesterol (mmol/L)	7·11 (0·68)	7·11 (0·67)
LDL-cholesterol (mmol/L)	4·70 (0·75)	4·69 (0·76)
HDL-cholesterol (mmol/L)	1·51 (0·44)	1·52 (0·46)
Triglyceride (mmol/L)*	1·74 (1·25–2·49)	1·73(1·23–2·48)
Blood pressure		
Systolic (mm Hg)	135 (21)	135 (21)
Diastolic(mm Hg)	79 (13)	79 (13)
HMG CoA RI		
Pravastatin	5553 (60%)	5523 (60%)
Simvastatin	3417 (37%)	3272 (36%)
Other statin	128 (1%)	110 (1%)
Medication use		
Antiplatelet agent	1342 (14%)	1258 (13%)
Calcium antagonist	2837 (30%)	2796 (30%)
β blocker	791 (8%)	794 (9%)
Other antihypertensive agents	2424 (26%)	2366 (25%)
Nitrate	926 (10%)	863 (9%)
Hypoglycaemic agents	1126 (12%)	1081 (12%)

Data are number of patients (%) or mean (SD), unless otherwise indicated. CABG=coronary-artery bypass grafting. PTCA=percutaneous transluminal coronary angioplasty. LDL=low-density lipoprotein. HDL=high-density lipoprotein. IQR=interquartile range. HMG CoA RI=3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor. BMI=body-mass index, which is weight in kg divided by the square of height in metres. *Median (IQR).

Table 1: **Baseline characteristics**

See Online for webtable

treatment allocation. The study was approved by an external data and safety monitoring board, by institutional review boards at all hospitals, and by regional organising committees. The data and safety monitoring board also monitored the rate of endpoints twice during the study, in March, 2002, and March, 2004. The study was followed up until November, 2004, because both interim analyses did not reach the stopping boundary.

We sampled blood to measure serum lipid at 6 and 12 months, and then every year until the final follow-up visits. Plasma total fatty acid concentrations for all patients who gave informed consent were measured with capillary gas chromatography every year at a central laboratory.

Statistical analysis

We used a two-sided test at the 5% significance level to estimate that the number of enrolled patients would give the study a statistical power of 80% for detection of a relative reduction of 25% in the primary endpoint rate, when the EPA group was compared with controls. The event rate of the primary endpoint in the control group was assumed to be 0·58% per year for primary prevention and 2·13% per year for secondary prevention; the proportion of primary and secondary prevention strata was assumed to be 4:1. The accrual period was assumed to be 3 years with a follow-up of 5 years for all patients. All analyses were based on the intention-to-treat principle. Time-to-event data were analysed with the Kaplan–Meier method and the log-rank test. The hazard ratio and its 95% confidence interval were computed with the Cox proportional hazard model. We did subgroup analyses with a model that included an interaction term corresponding to the test for heterogeneity in effects. Changes in lipid values were compared by repeated measures of ANOVA. Data were analysed with SAS statistical software (version 8.12).

Role of the funding source

The sponsor had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The JELIS steering committee had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Patients were monitored for an average of 4·6 years (SD 1·1). Table 1 shows baseline characteristics of the treatment groups. The mean age of all patients was 61 years and 12 786 patients (69%) were women. Mean concentrations of total cholesterol and triglyceride were 7·1 mmol/L and 1·7 mmol/L; and mean LDL and HDL cholesterol concentrations were 4·7 mmol/L and 1·5 mmol/L, respectively. The webtable shows baseline characteristics for primary and secondary prevention subgroups. Of 3664 patients with documented coronary artery disease, 1050 had a history of myocardial infarction, 2903 of angina pectoris, and 895 angioplasty, stenting, or coronary artery bypass grafting.

Average doses were pravastatin 10·0 mg daily (SD 9·1) and simvastatin 5·6 mg daily (1·8). 16 449 (90%) patients took 10 mg pravastatin or 5 mg simvastatin. The 5-year follow-up rate was 16 971 (91%). Similar proportions of participants remained compliant in each treatment group. Study drug regimens were maintained until trial termination by 6151 (73%) of controls and in the treatment group 5883 (71%) of patients continued to take EPA and 6136 (74%) continued to take statin.

586 patients (262 assigned to EPA and 324 controls) reached the composite primary endpoint. Figure 2 shows Kaplan–Meier curves for the primary endpoint. The 5-year cumulative rate of major coronary events

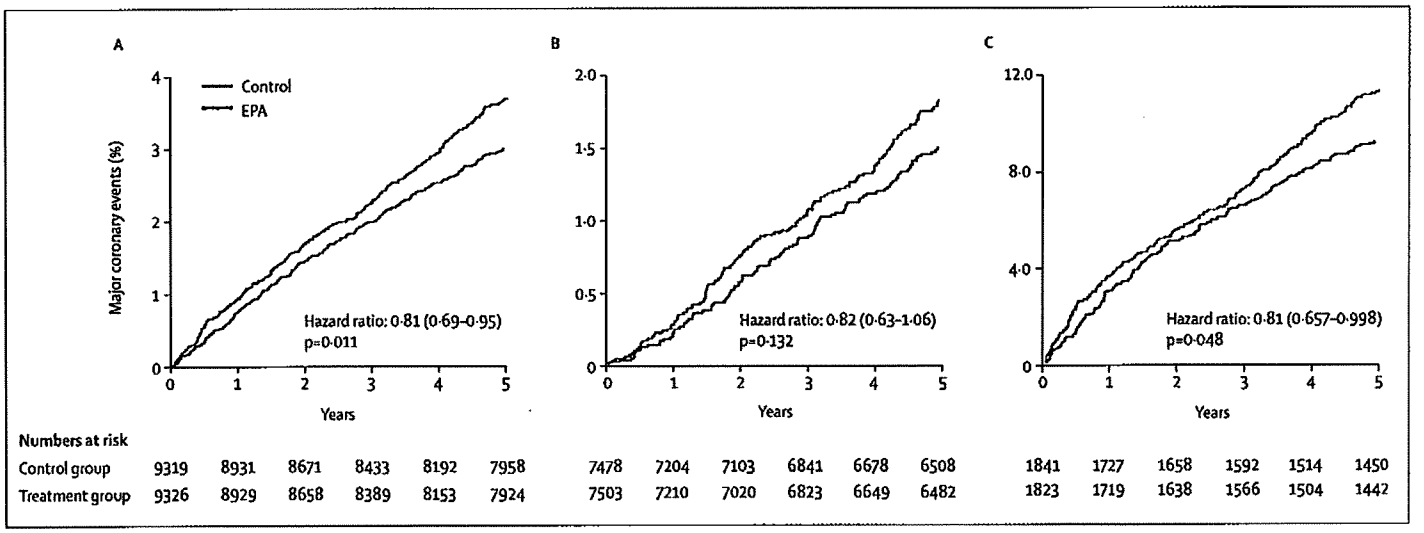

Figure 2: Kaplan-Meier estimates of incidence of coronary events in the total study population (panel A), the primary prevention arm (panel B) and the secondary prevention arm (panel C)

was 2·8% in the EPA group and 3·5% in controls, resulting in a significant relative risk reduction of 19% in the EPA group (p=0·011). Figure 3 shows that EPA treatment was associated with a significant reduction of 24% in the frequency of unstable angina. The occurrence of coronary death or myocardial infarction was not significantly lower (22%) in the EPA group than in controls. The frequency of fatal or non-fatal myocardial infarction was not significantly reduced (23%) in the EPA group; however, that of non-fatal coronary events (including non-fatal myocardial infarction, unstable angina, and events of angioplasty, stenting, or coronary artery bypass grafting) was significantly lower (19%) in the EPA group than in controls.

Table 2 sets out major coronary events in the two treatment groups for comparison with specific background characteristics of all populations. For example, we grouped patients according to their LDL cholesterol at baseline. The relative reduction in major coronary events risk in the EPA group was of a similar magnitude in patients with different ranges of LDL cholesterol values, suggesting that LDL cholesterol is not an important factor in reduction of risk for major coronary events.

In the primary prevention subgroup, EPA treatment was associated with a non-significant 18% reduction in major coronary events. Figure 3 shows the non-significant reductions of 18%, 21%, and 20% in coronary death or non-fatal myocardial infarction, fatal or non-fatal myocardial infarction, and non-fatal coronary events, respectively. In the secondary prevention subgroup, allocation to the EPA treatment was associated with a significant 19% reduction in major coronary events. EPA treatment was also associated with a significant 28% reduction in the incidence of unstable angina. This treatment also produced non-significant reductions of 25%, 25%, and 18% in coronary death or myocardial infarction, fatal or non-fatal myocardial infarction, and non-fatal coronary events, respectively.

In the other analyses, stroke occurred in 162 (1·7%) controls and 166 (1·8%) patients given EPA. Figure 3 shows that the frequency of ischaemic and haemorrhagic strokes did not differ between the two treatment groups, and neither did all-cause mortality.

Figure 4 summarises the change in lipid values after treatment. Total and LDL cholesterol at the last clinic visit decreased significantly by 19% and 25% from baseline in both groups, respectively. Triglyceride decreased significantly by 9% from baseline in the EPA group and by 4% in controls (p<0·0001 between groups). Both treatments produced only small changes in HDL cholesterol. The fatty acid concentrations at baseline were the average values for all patients who gave informed consent in the control group (n=8076) and the EPA group (n=8321). Plasma EPA at baseline was 2·9% of total molecules of fatty acids (mol %). To assess the effect of EPA treatment, plasma fatty acid values were compared for all patients who were still compliant after 5 years of observation (controls: n=4854, EPA group: n=4970). Plasma EPA concentration and the ratio of EPA to arachidonic acid at baseline were 93 mg/L and 0·60 in controls, and 97 mg/L and 0·63 in the EPA group, respectively. Plasma EPA concentration and the ratio of EPA to arachidonic acid at year 5 were 93 mg/L and 0·59 in controls. On the other hand, plasma EPA concentration at year 5 was 169 mg/L in the EPA group, which was a 70% increase from baseline. The ratio of EPA to arachidonic acid increased two-fold from 0·63 to 1·23 in the EPA group. Similar results were reported previously.[11,28]

Table 3 shows that a quarter of patients in the EPA group had adverse experiences related to treatment, compared with about a fifth of controls. Rates of

Event	Number (%) of patients: Control	EPA	p value	Hazard ratio (95 %CI)
All patients				
Major coronary events	324 (3·5)	262 (2·8)	0·011	0·81 (0·69–0·95)
Items of account				
Sudden cardiac death	17 (0·2)	18 (0·2)	0·854	1·06 (0·55–2·07)
Fatal MI	14 (0·2)	11 (0·1)	0·557	0·79 (0·36–1·74)
Non-fatal MI	83 (0·9)	62 (0·7)	0·086	0·75 (0·54–1·04)
Unstable angina	193 (2·1)	147 (1·6)	0·014	0·76 (0·62–0·95)
CABG or PTCA	222 (2·4)	191 (2·1)	0·135	0·86 (0·71–1·05)
Combined endpoint				
Coronary death or MI	113 (1·2)	88 (0·9)	0·083	0·78 (0·59–1·03)
Fatal MI or nonfatal MI	93 (1·0)	71 (0·8)	0·091	0·77 (0·56–1·05)
Coronary death	31 (0·3)	29 (0·3)	0·812	0·94 (0·57–1·56)
Non-fatal coronary events	297 (3·2)	240 (2·6)	0·015	0·81 (0·68–0·96)
Primary prevention of CAD				
Major coronary events	127 (1·7)	104 (1·4)	0·132	0·82 (0·63–1·06)
Items of account				
Sudden cardiac death	4 (0·1)	5 (0·1)	0·736	1·25 (0·34–4·67)
Fatal MI	6 (0·1)	6 (0·1)	0·995	1·00 (0·32–3·11)
Non-fatal MI	45 (0·6)	36 (0·5)	0·321	0·80 (0·52–1·24)
Unstable angina	70 (0·9)	59 (0·8)	0·338	0·85 (0·60–1·19)
CABG or PTCA	74 (1·0)	64 (0·9)	0·400	0·87 (0·62–1·21)
Combined endpoint				
Coronary death or MI	55 (0·7)	45 (0·6)	0·322	0·82 (0·55–1·22)
Fatal MI or nonfatal MI	51 (0·7)	40 (0·5)	0·253	0·79 (0·52–1·19)
Coronary death	10 (0·1)	11 (0·1)	0·822	1·10 (0·47–2·60)
Non-fatal coronary events	119 (1·6)	95 (1·3)	0·102	0·80 (0·61–1·05)
Secondary prevention of CAD				
Major coronary events	197 (10·7)	158 (8·7)	0·048	0·81 (0·66–1·00)
Items of account				
Sudden cardiac death	13 (0·7)	13 (0·7)	0·967	1·02 (0·47–2·19)
Fatal MI	8 (0·4)	5 (0·3)	0·421	0·64 (0·21–1·94)
Non-fatal MI	38 (2·1)	26 (1·4)	0·150	0·70 (0·42–1·14)
Unstable angina	123 (6·7)	88 (4·8)	0·019	0·72 (0·55–0·95)
CABG or PTCA	148 (8·0)	127 (7·0)	0·243	0·87 (0·69–1·10)
Combined endpoint				
Coronary death or MI	58 (3·2)	43 (2·4)	0·156	0·75 (0·51–1·12)
Fatal MI or nonfatal MI	42 (2·3)	31 (1·7)	0·223	0·75 (0·47–1·19)
Coronary death	21 (1·1)	18 (1·0)	0·667	0·87 (0·46–1·64)
Non-fatal coronary events	178 (9·7)	145 (8·0)	0·080	0·82 (0·66–1·02)
Other analyses				
Stroke	162 (1·7)	166 (1·8)	0·785	1·02 (0·91–1·13)
Ischaemic	123 (1·3)	115 (1·2)	0·632	0·97 (0·85–1·10)
Haemorrhagic	39 (0·4)	49 (0·5)	0·272	1·12 (0·91–1·39)
Other type or not determined	5 (0·1)	2 (<0·1)	0·252	0·63 (0·24–1·37)
All-cause death	265 (2·8)	286 (3·1)	0·333	1·09 (0·92–1·28)

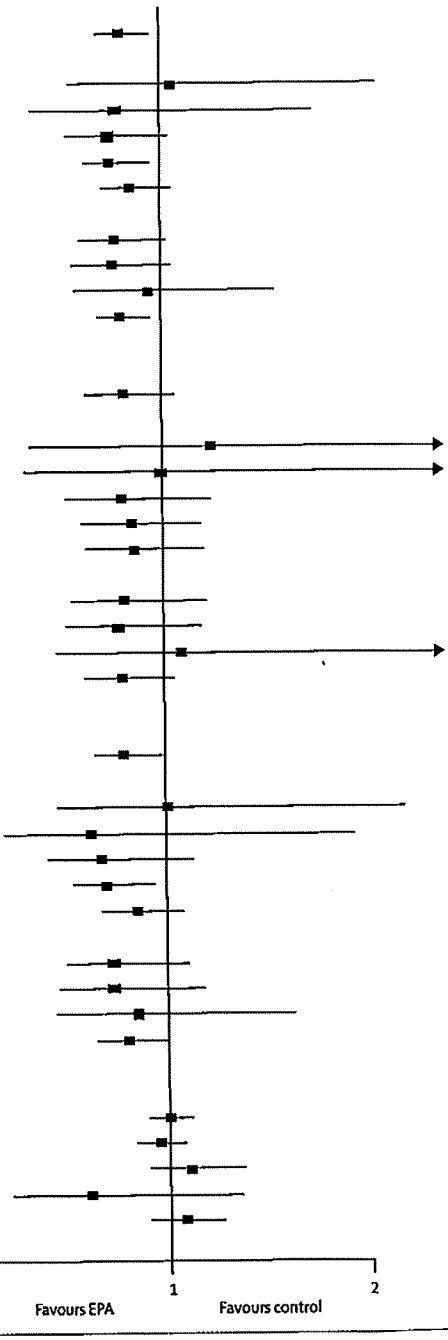

Figure 3: **Estimated hazard ratios of clinical endpoints stratified by prevention stratum**
MI=myocardial infarction. CABG=coronary-artery bypass grafting. PTCA=percutaneous transluminal coronary angioplasty. CAD=coronary-artery disease.

discontinuation because of treatment-related adverse events were 1087 (11·7%) in the EPA group and 673 (7·2%) in the control group. Most adverse effects attributable to EPA allocation were regarded as mild. The following factors were more common in the EPA group than in controls: abnormal laboratory data; gastrointestinal disturbances such as nausea, diarrho or epigastric discomfort; skin abnormalities such eruption, itching, exanthema, or eczema; a haemorrhages such as cerebral and fundal bleedin epistaxis, and subcutaneous bleeding. The frequency new cancers did not differ.

Discussion

Our results show that EPA treatment reduced the frequency of major coronary events. The composite frequency of the primary endpoint in all patients for the EPA group was 19% lower than in controls. The risks of unstable angina and non-fatal coronary events were also substantially reduced, by 24% and 19%, respectively. The beneficial effects of EPA seemed much the same in both the secondary prevention and the primary prevention subgroups, although they were significant only in the EPA group because of greater numbers of events.

We showed that the reduced risk associated with EPA treatment was confined to non-fatal coronary events. However, the reduced risk did not apply to coronary death or sudden cardiac death in any of our study populations or secondary prevention subgroup studies. This finding differs from the results of previous interventional and observational studies.[29] Most observational studies report that fish intake only once or twice a week or a small intake of fish about 30–60 g per day is associated with a 30–60% reduction in the risk of fatal coronary events or sudden cardiac deaths, but not of non-fatal coronary events.[1,3–5,7] Secondary prevention trials for coronary heart disease report that a modest intake of fatty fish (200–400 g/week) or supplemental intake of EPA plus DHA (1 g/d) reduces coronary mortality by about 20–30% in patients who have already had a myocardial infarction.[8,9] Experimental and epidemiological studies suggest that fish oil at low doses might prevent sudden cardiac death by an antiarrhythmic effect.[30]

Our findings accord with a cohort study by the Japan Public Health Centre, which used a food-frequency questionnaire.[31] Iso and co-workers[31] reported that, compared with a small intake of fish (once a week or about 20 g per day), a high intake (eight times per week, or about 180 g per day) was associated with a substantially reduced risk of coronary heart disease, especially non-fatal cardiac events, in middle-aged Japanese men and women. This finding suggests that two protective mechanisms of EPA or n-3 polyunsaturated fatty acids affect the risk of coronary events: reduction of mortality from coronary artery disease and sudden cardiac death with a low intake of n-3 polyunsaturated fatty acid, and reduction of all coronary events with a high intake of n-3 polyunsaturated fatty acids. Our patients could possibly all have had intakes of fish that were above the threshold for prevention of fatal coronary events or sudden cardiac death.[4] One potential explanation for the strong inverse association with non-fatal coronary events in our study population, but not in other study populations of non-Japanese patients, is that EPA might affect risk only at very high levels of fish intake, such as those common in Japan.

n-3 polyunsaturated fatty acids have antiarrhythmic effects and other beneficial effects,[32,33] such as reduced

	Control (n=9319)	EPA (n=9326)	Hazard ratio (95% CI)	Interaction p
Age (years)				
<61	117/4380 (2·7)	87/4275 (2·0)	0·76 (0·57–1·00)	0·57
≥61	207/4939 (4·2)	175/5051 (3·5)	0·84 (0·68–1·02)	
Sex				
Female	126/6411 (2·0)	109/6375 (1·7)	0·87 (0·68–1·13)	0·43
Male	198/2908 (6·8)	153/2951 (5·2)	0·76 (0·62–0·94)	
BMI				
<24	136/4404 (3·1)	109/4386 (2·5)	0·80 (0·62–1·03)	0·88
≥24	148/4021 (3·7)	123/4078 (3·0)	0·82 (0·65–1·05)	
Previous CAD				
Absent	127/7478 (1·7)	104/7503 (1·4)	0·82 (0·63–1·06)	0·95
Present	197/1841 (10·7)	158/1823 (8·7)	0·81 (0·66–1·00)	
Smoking				
Non-smoker	216/7619 (2·8)	170/7496 (2·3)	0·80 (0·66–0·98)	0·89
Smoker	108/1700 (6·4)	92/1830 (5·0)	0·78 (0·59–1·04)	
Diabetes				
Absent	221/7795 (2·8)	175/7810 (2·2)	0·79 (0·65–0·96)	0·62
Present	103/1524 (6·8)	87/1516 (5·7)	0·86 (0·65–1·15)	
Hypertension				
Absent	167/6037 (2·8)	139/5997 (2·3)	0·85 (0·68–1·06)	0·57
Present	157/3282 (4·8)	123/3329 (3·7)	0·77 (0·61–0·97)	
Total cholesterol (mmol/L)				
<7·0	167/4700 (3·6)	145/4751 (3·1)	0·86 (0·69–1·08)	0·46
≥7·0	156/4608 (3·4)	117/4550 (2·6)	0·76 (0·60–0·97)	
Triglyceride (mmol/L)				
<1·7	130/4555 (2·9)	105/4635 (2·3)	0·79 (0·61–1·02)	0·75
≥1·7	188/4648 (4·0)	153/4563 (3·4)	0·84 (0·68–1·04)	
HDL-cholesterol (mmol/L)				
<1·5	206/4316 (4·8)	154/4149 (3·7)	0·78 (0·64–0·96)	0·26
≥1·5	91/4285 (2·1)	91/4491 (2·0)	0·96 (0·72–1·28)	
LDL-cholesterol (mmol/L)				
<4·7	129/4160 (3·1)	108/4251 (2·5)	0·82 (0·64–1·06)	0·83
≥4·7	156/4157 (3·8)	131/4097 (3·2)	0·86 (0·68–1·08)	

Data are number of patients (%) or hazard ratio (95% CI). p values are for the test of heterogeneity. CAD=coronary artery disease. BMI=body-mass index, which is weight in kg divided by the square of height in metres. LDL=low-density lipoprotein. HDL=high-density lipoprotein. There is a deficit of clinical data in some patients with the events.

Table 2: **Subgroup analysis**

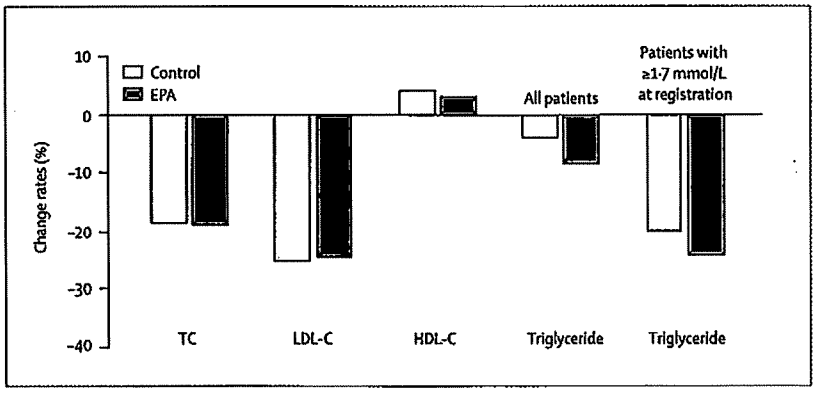

Figure 4: **Percentage changes from baseline in serum lipid profile**
TC=total cholesterol. LDL C=low-density lipoprotein cholesterol. HDL C=high-density lipoprotein cholesterol.

	Control (n=9319)	EPA (n=9326)	p value
Total number of adverse experiences (%)	2004 (21·7%)	2334 (25·3%)	<0·0001
Newly diagnosed cancer			
Total	218 (2·4%)	242 (2·6%)	0·26
Stomach	37 (0·4%)	53 (0·6%)	0·09
Lung	37 (0·4%)	32 (0·3%)	0·54
Colorectal	29 (0·3%)	26 (0·3%)	0·68
Breast	21 (0·2%)	16 (0·2%)	0·41
Common adverse experiences			
Pain (joint pain, lumbar pain, muscle pain)	180 (2·0%)	144 (1·6%)	0·04
Gastrointestinal disturbance (nausea, diarrhoea, epigastric discomfort)	155 (1·7%)	352 (3·8%)	<0·0001
Skin abnormality (eruption, itching, exanthema, eczema)	65 (0·7%)	160 (1·7%)	<0·0001
Haemorrhage (cerebral, fundal, epistaxis, subcutaneous)	60 (0·6%)	105 (1·1%)	0·0006
Abnormal laboratory data			
Total	322 (3·5%)	378 (4·1%)	0·03
CPK increased	116 (1·2%)	126 (1·4%)	0·52
GOT increased	38 (0·4%)	59 (0·6%)	0·03
Sugar blood level increased	27 (0·3%)	38 (0·4%)	0·17

CPK=creatine phosphokinase. GOT=glutamic oxaloacetic transaminase.

Table 3: Adverse experiences

platelet aggregation,[10,11] vasodilation,[12,13] antiproliferation,[14] plaque-stabilisation,[15] and a reduction in lipid action.[16,17] One clinical study examined the morphology of endoatherectomised carotid specimens and showed that fish-oil supplementation increased the stability of atherosclerotic plaque.[15,34] Atherosclerotic plaque is vulnerable to rupture because it has a thin fibrous cap that covers a large lipid core, and an increased number of inflammatory cells such as macrophages. n-3 polyunsaturated fatty acids reduce the expression of adhesion molecules on endothelial cells[35] and macrophages.[36] Dietary fish oil reduces the production of chemoattractants, including leukotriene B4,[37] platelet-derived growth factor,[38] and monocyte chemoattractant protein-1.[39] These mechanisms reduce the passage of monocytes and macrophages into the plaque. Thus, EPA and DHA reduce the numbers of macrophages in the atherosclerotic plaque. Thrombus formation in the ruptured plaque leads to acute cardiovascular events.

Our study has some specific characteristics. First, we used highly purified EPA rather than n-3 polyunsaturated fatty acids or fish oils. This trial is a pharmacological intervention rather than a food-based or nutrient trial. Nutritional data are difficult to extrapolate to pharmacological intervention because fish oil contains many fatty acids other than EPA and DHA. Although both EPA and DHA are biologically active, we do not know whether they have differential effects on cardiovascular protection. Second, our population was exclusively Japanese. In Japan, death from coronary artery disease is rare and the average dietary intake of fish is about five times higher than that in other countries.[28] We did not use a food-frequency questionnaire to measure fish intake; instead, at baseline, we measured plasma fatty acid concentrations that indicate fish consumption and EPA intake. Plasma EPA was 2·9 mol% at baseline in our study population, which is similar to reports by Iso and co-workers[40] that serum EPA composition was 4·1 mol% in rural Japanese and 2·4 mol% in urban Japanese; these values are much higher than those recorded in the USA, which are about 0·3 mol%.

Our trial has several limitations. First, we used an open interventional design, with blinded clinical endpoint assessment (PROBE design) to keep bias to a minimum.[23] The PROBE design has the advantages of low costs and similarity to standard clinical practice, which should make the results easily applicable in routine medical care; however, we cannot exclude the possibility of bias in some of the physician-initiated endpoints, such as coronary revascularisation and hospital treatment for unstable angina.

Second, we prescribed either pravastatin or simvastatin for all participants as the first-line treatment, in part because these were the two statins available in the Japanese market at the start of this study. We used the low doses of statins that are recommended by Japan's Ministry of Health, Labour, and Welfare. Such low doses have been reported to control serum lipid concentrations and major coronary events in Japanese patients.[41,42] We did not use a true placebo group.

Third, this trial was substantially underpowered for analysis of subgroups. Death associated with coronary artery disease in the Japanese population is about 22–26 per 100 000 person-years, which is very low in comparison with that in the USA and northern Europe.[28] This difference is thought to be partly due to differences in dietary habits, including fish consumption. About two-thirds of patients in our study were women, who have an incidence of coronary events that is 2·3 times lower than that for men.[41] This low ratio of men to women and the Japanese study population could have contributed to the overall low rate of coronary events, including coronary death, which failed to detect a significant effect on primary prevention outcomes.

Studies show that use of high-dose statin treatment can produce an extra reduction in cardiac events, by achievement of the maximum lowering of LDL cholesterol.[43] Similar benefits could arguably be obtained if the dose of statin was increased without the addition of EPA; however, we noted that EPA did not affect LDL cholesterol concentrations and that this 19% reduction in major coronary events in the EPA group was not related to serum LDL cholesterol. This finding suggests that EPA exerts its effects via mechanisms that are independent of a reduction in LDL cholesterol.

We adopted the most widely used therapeutic dose of EPA (1800 mg per day), which is approved by Japan's Ministry of Health, Labour, and Welfare. We noted no significant difference in all-cause mortality between the treatment and control groups. There was no difference in the rate of cancer and stroke, including cerebral bleeding, subarachnoidal bleeding, or both. We do not know whether lower or higher doses of EPA would produce different effects from those noted at the dose used in our study.

The beneficial effects of EPA could have stemmed from many biological effects that lead to the attenuation of thrombosis, inflammation, and arrhythmia in addition to a reduction of triglycerides. Overall, this study shows that EPA, at a dose of 1800 mg per day, is a very promising regimen for prevention of major coronary events, especially since EPA seems to act through several biological mechanisms. Because our population was exclusively Japanese, we cannot generalise our results to other populations. We need to investigate whether EPA is effective for prevention of major coronary events in hypercholesterolaemic patients without or with coronary artery disease in other countries.

Contributors

Investigators on the steering committee of the study designed, conducted, analysed, and interpreted the present study. A statistical coordination centre collected, managed, and analysed the data. All authors have participated in the data analysis and reporting stage of this manuscript. The principal investigator prepared the first draft, and all members of the JELIS Steering Committee contributed to writing, and have seen and approved the final version.

Conflict of interest statement

The committee members and investigators received no remuneration for conducting this study. M Yokoyama received travel costs from Mochida Pharmaceutical Co Ltd, Tokyo, Japan, to participate in the scientific meeting. Other authors have no conflicts of interest.

Acknowledgments

This study was supported by grants from Mochida Pharmaceutical Co Ltd, Tokyo, Japan. The results were presented in part at the late-breaking clinical trials of the American Heart Association Annual Meeting, Dallas, TX, USA, Nov, 13–16, 2005. We thank all trial participants and the large numbers of doctors, nurses, and hospital staff who made long-term commitments to the study.

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Journal of Neurochemistry, 2007, **102**, 1595–1605 doi:10.1111/j.1471-4159.2007.04685.x

Enhancement of α-secretase cleavage of amyloid precursor protein by a metalloendopeptidase nardilysin

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Abstract

Amyloid-β (Aβ) peptide, the principal component of senile plaques in the brains of patients with Alzheimer's disease, is derived from proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases. Alternative cleavage of APP by α-secretase occurs within the Aβ domain and precludes generation of Aβ peptide. Three members of the ADAM (a disintegrin and metalloprotease) family of proteases, ADAM9, 10 and 17, are the main candidates for α-secretases. However, the mechanism that regulates α-secretase activity remains unclear. We have recently demonstrated that nardilysin (EC 3.4.24.61, *N*-arginine dibasic convertase; NRDc) enhances ectodomain shedding of heparin-binding epidermal growth factor-like growth factor through activation of ADAM17. In this study, we show that NRDc enhances the α-secretase activity of ADAMs, which results in a decrease in the amount of Aβ generated. When expressed with ADAMs in cells, NRDc dramatically increased the secretion of α-secretase-cleaved soluble APP and reduced the amount of Aβ peptide generated. A peptide cleavage assay *in vitro* also showed that recombinant NRDc enhances ADAM17-induced cleavage of the peptide substrate corresponding to the α-secretase cleavage site of APP. A reduction of endogenous NRDc by RNA interference was accompanied by a decrease in the cleavage by α-secretase of APP and increase in the amount of Aβ generated. Notably, NRDc is clearly expressed in cortical neurons in human brain. Our results indicate that NRDc is involved in the metabolism of APP through regulation of the α-secretase activity of ADAMs, which may be a novel target for the treatment of Alzheimer's disease.

Keywords: a disintegrin and metalloprotease proteases, α-secretase, Alzheimer's disease, amyloid-β, ectodomain shedding, nardilysin.

J. Neurochem. (2007) **102**, 1595–1605.

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is characterized by a variety of pathological features in the brain such as extracellular senile plaques and intracellular neurofibrillary tangles. The main component of the extracellular senile plaques is amyloid β (Aβ) peptide, which is derived from amyloid precursor protein (APP), a type I transmembrane protein, by two sequential proteolytic cleavages. The initial cleavage is mediated by β-secretase, β-site APP-cleaving enzyme 1, at the N-terminus of the Aβ domain, which generates a soluble N-terminal fragment (β-secretase-cleaved soluble APP; sAPPβ) and a transmembrane C-terminal fragment (C99). C99 is susceptible to a second intramembrane cleavage by γ-secretase, releasing the 4-kDa

Received January 16, 2007; revised manuscript received March 27, 2007; accepted April 19, 2007.

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Abbreviations used: AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; APP_{SWE}, Swedish mutant APP; Aβ, amyloid β; C83, C-terminal fragment of 83 amino acids of APP; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; HB-EGF, heparin-binding epidermal growth factor-like growth factor; IDE, insulin-degrading enzyme; N2a, Neuro2a; NRDc, *N*-arginine dibasic convertase; sAPP, secretase-cleaved soluble APP; TACE, tumor necrosis factor-α converting enzyme; TNF-α, tumor necrosis factor-α; TOF, time-of-flight.

Aβ peptide (Sinha *et al.* 1999; Vassar *et al.* 1999; Selkoe 2001; De Strooper 2003; Haass 2004). γ-Secretase is a multiprotein complex consisting of presenilin-1, nicastrin, anterior pharynx defective-1, and presenilin enhancer-2 (De Strooper 2003; Takasugi *et al.* 2003).

In addition to the amyloidgenic pathway, APP is cleaved within the Aβ domain by α-secretase, which precludes the formation of Aβ peptides. This cleavage generates a secreted ectodomain of APP (sAPPα) and a C-terminal fragment of 83 amino acids of APP (C83). C83 can be further cleaved by γ-secretase to yield a 3-kDa fragment (p3) of Aβ, which is not found in the amyloid cores of senile plaques (Haass *et al.* 1993; Naslund *et al.* 1994). sAPPα appears to have not only neuroprotective properties (Small *et al.* 1994; Furukawa *et al.* 1996) but also memory-enhancing effects (Meziane *et al.* 1998). Therefore, enhancement of α-secretase activity might be beneficial for the treatment of AD. Several members of the ADAM (a disintegrin and metalloprotease) family of proteases such as ADAM9, ADAM10, and ADAM17/tumor necrosis factor-α (TNF-α) converting enzyme (TACE) have been reported to have α-secretase activity (Buxbaum *et al.* 1998; Koike *et al.* 1999; Lammich *et al.* 1999; Hooper and Turner 2002; Allinson *et al.* 2003). Fibroblasts derived from TACE-deficient mice fail to secrete sAPPα in response to phorbol ester, while the basal secretion of sAPPα is unaffected in TACE-deficient cells (Buxbaum *et al.* 1998). Over-expression of ADAM10 in human embryonic kidney cells 293 increases basal and phorbol ester-stimulated α-secretase activity, while the basal and stimulated α-secretase activity is inhibited by the over-expression of an enzymatically inactive form of ADAM10 (Lammich *et al.* 1999). Furthermore, a recent report has shown the first evidence that ADAM protease acts as an α-secretase *in vivo* (Postina *et al.* 2004). In an animal model of AD, in which human APP is over-expressed in the brains of mice, neuronal over-expression of ADAM10 prevented senile plaques from forming, accompanied by an increase in the secretion of sAPPα and a reduction in the amount of Aβ generated (Postina *et al.* 2004). In contrast, over-expression of the inactive mutant of ADAM10 in the brain enhanced the formation of senile plaques (Postina *et al.* 2004). Further studies will be required, however, to specify the physiologically relevance of α-secretases and to understand the regulation of α-secretase activity *in vivo*.

Nardilysin (EC 3.4.24.61, *N*-arginine dibasic convertase; NRDc) was initially cloned as a zinc metalloendopeptidase of the M16 family, which can selectively cleave the dibasic site *in vitro* (Chesneau *et al.* 1994). We have reported that NRDc binds specifically to heparin-binding epidermal growth factor-like growth factor (HB-EGF) among EGF family members, which enhances HB-EGF-induced cell migration (Nishi *et al.* 2001). We also recently reported that NRDc enhances ectodomain shedding of HB-EGF through the activation of TACE (Nishi *et al.* 2006). NRDc binds to and directly enhances the catalytic activity of TACE, which was the first description of how ectodomain shedding is regulated by the modulation of sheddase activity (Nishi *et al.* 2006). TACE is involved in the ectodomain shedding of a wide variety of membrane proteins, including TNF-α (Black *et al.* 1997), APP (Buxbaum *et al.* 1998), transforming growth factor-α (Peschon *et al.* 1998), interleukin-6 receptor (Althoff *et al.* 2000), L-selectin (Peschon *et al.* 1998), and Notch 1 receptor (Brou *et al.* 2000). To test whether NRDc enhances the ectodomain shedding of other TACE substrates, as well as HB-EGF, we examined the effect of NRDc on the cleavage of APP by α-secretase. In this study, we demonstrate that NRDc enhances TACE-induced α-cleavage of APP, which results in a reduction in the production of Aβ.

Materials and methods

Plasmids

Expression plasmids for human NRDc (pcDNA3.1-hNRDc-V5), the enzymatically inactive mutant of human NRDc (pcDNA3.1-hNRDc E235A-V5), and human TACE (pME18S-hTACE) were described previously (Nishi *et al.* 2006). The human cDNA for ADAM10 was cloned into pME18S to generate pME18S-hADAM10. Expression plasmids for human ADAM9 and ADAM12 and for human APP_{770} (APP_{WT}) were generously provided by A. Sehara and by A. Kinoshita, respectively.

Site-directed mutagenesis of the human APP gene

The Swedish mutant APP (APP_{SWE}) is a familial AD-associated mutant form of APP, in which two amino acids immediately flanking the N-terminus of the Aβ domain (lysine and methionine) are mutated to asparagine and leucine (APP_{SWE}) (Forman *et al.* 1997). To generate APP_{SWE}, site-directed mutagenesis of the human APP_{770} gene was performed by using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). A single set of oligonucleotides was designed as follows: sense 5′-CGGAGGAGATCTCTGAAGTGA ACTTGGATGCAGAATTCCG-3′; antisense 5′-CGGAATTCTGC ATCCAAGTTCACTTCAGAGATCTCCTCCG-3′. Mutations were confirmed by DNA sequencing. The underlined codons encode the mutated amino acids.

RNA interference

The sequences for siRNA duplexes against mouse NRDc, human TACE and human ADAM10 were as follows: mNRDc1, 5′-AAG GGUGAAGCCACUUCAUUA-3′; mNRDc2, 5′-GAUAAUGCCU CAACUGAUUUU-3′; TACE-1, 5′-AUGAGUUGUAACCAGGU CAGCUUCC-3′; TACE-2, 5′-AUACAUGACAUAUUUCCCU CCCUGG-3′; ADAM10-1, 5′-UACACCAGUCAUCUGGUAU UUCCUC-3′; ADAM10-2, 5′-AGAAUUAACACUCUCAGCAAC ACCA-3′. siRNA duplexes against NRDc were synthesized by Dharmacon, Lafayette, CO, USA. siRNA duplexes against TACE and ADAM10 were purchased from Invitrogen (Carlsbad, CA, USA). Cy3-conjugated control siRNA (ATCCGCGCGATAGTAC GTA; B-Bridge International, Sunnyvale, CA, USA) was used as a transfection efficiency detector and a negative control.

Cell culture and transfections

COS7, 293T, and Neuro2a (N2a) cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine at 37°C in a humidified incubator with 5% CO_2. Transfections were carried out using FuGENE 6 (Roche Diagnostics, Mannheim, Germany) for plasmids and siFECTOR (B-Bridge International) for siRNA, according to the manufacturer's instructions.

Western blot analysis

Cells were lysed in lysis buffer containing 10 mmol/L Tris–HCl (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40, and protease inhibitor cocktail (Complete Mini; Roche Diagnostics). Cell lysates were collected after centrifugation at 16 000 *g* for 10 min. Equivalent amounts of protein were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4–12% gradient Bis–Tris gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies followed by an appropriate peroxidase-conjugated secondary antibody and visualized with the enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire, UK). Anti-APP antibody (A8717; Sigma, St Louis, MO, USA) was used to recognize the full-length version and the C83 of APP (Xie *et al.* 2005). Anti-V5 tag (Invitrogen) and mouse monoclonal antibody for NRDc (#23 or #304) were used to recognize V5-tagged NRDc and endogenous NRDc, respectively (Nishi *et al.* 2006). Anti-TACE (C-15) antibody, anti-ADAM10 (Ab-1) antibody, and anti-glyceraldehyde-3 phosphate dehydrogenase antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Calbiochem (San Diego, CA, USA), and Research Diagnostics (Concord, MA, USA), respectively.

α-Secretase cleavage assay

COS7 or 293T cells were plated on six-well culture plates (approximately 50% confluent) and transiently co-transfected with APP_{WT} or APP_{SWE} in combination with the indicated ADAM protease and/or NRDc. Cells were washed with serum-free media (Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin) and incubated in the same media 24 h post-transfection. After an additional 4 h of incubation, the conditioned medium and total cell lysate were collected. The amount of the soluble form of APP cleaved by α-secretase (sAPPα) in the conditioned medium was measured by ELISA (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. The C-terminal remnant of APP was detected by western blotting as described above. For secretase inhibitor experiments, the metalloprotease inhibitor TAPI-2 (100 μmol/L in ethanol; Calbiochem), β-secretase inhibitor II (15 μmol/L in dimethylsulfoxide; Calbiochem), or γ-secretase inhibitor (70 μmol/L in dimethylsulfoxide; Sigma) was incubated with transfected cells for 4 h before the collection of the conditioned medium and total cell lysate.

Detection of secreted Aβ by sandwich ELISA

The Aβ present in conditioned medium was detected by sandwich ELISA, using anti-Aβ antibody, 6E10 (Sigma) as the capture antibody and anti-$Aβ_{40}$ antibody conjugated to biotin (Novus Biologicals, Littleton, CO, USA) as the detection antibody. After incubation with streptavidin-horseradish peroxidase, the reaction was developed with the tetramethylbenzidine peroxidase substrate system (R&D Systems). The Aβ values were quantified using standard curves prepared with synthetic $Aβ_{40}$ (Sigma).

Peptide cleavage assay and molecular mass analysis

The internally quenched fluorogenic peptide substrate containing the α-secretase cleavage site of APP, α-secretase substrate II (100 μmol/L; Calbiochem), was incubated with recombinant TACE (5 μg/mL; R&D Systems) and/or recombinant NRDc (5 μg/mL) (Nishi *et al.* 2006) in a reaction buffer (25 mmol/L Tris–HCl pH 9.0 and 2.5 mmol/L $ZnCl_2$). After 15 h of incubation at 37°C, the fluorescence intensity was measured by a plate reader (Wallac 1420 ARVOsx; Perkin Elmer, Waltham, MA, USA) with excitation/emission wavelengths of 340/460 nm. To confirm the cleavage site, 100 μmol/L of $Aβ_{40}$ peptide (Sigma) was incubated in the reaction buffer in combination with recombinant NRDc (25 μg/mL) and/or TACE (5 μg/mL) for 15 h at 37°C. The $Aβ_{40}$ peptide after the incubation was analyzed by matrix-assisted laser desorption/ionization time-of-flight (TOF) mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics, Billerica, MA, USA).

Immunochemical staining

Tissue materials were obtained from autopsied brains collected at the Neuropathology Laboratories of Kyoto University, Kyoto, Japan. Tissue sections were prepared as described previously (Kawamoto *et al.* 2006). Standard histological methods including silver impregnation by Bielschowsky method were performed on paraffin sections. For immunohistochemical staining of NRDc, mouse monoclonal antibody for NRDc (#304), which was raised against recombinant human NRDc in our laboratory, was used as a primary antibody (8 μg/mL IgG). Bound antibodies were detected by horseradish peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare) and diaminobenzidine tetrahydrochloride as a chromogen. After the detection, sections were counter-stained with Mayer's hematoxylin solution. Immunocytochemical staining of COS7 cells transiently transfected with NRDc-V5 was performed as described previously (Nishi *et al.* 2006). All procedures for autopsied brains were in accordance with institutional guidelines, and informed consent was obtained from relatives of all subjects.

Results

NRDc enhances the α-secretase activity of TACE

To examine the effect of NRDc on the α-secretase activity of TACE, human APP_{770} (APP_{WT}) was transiently introduced into COS7 cells or 293T cells with NRDc and/or TACE. The amounts of the soluble form of APP (sAPPα) in conditioned medium and the C-terminal remnant of APP (C83) in cell lysates were detected by ELISA (Fig. 1a) and immunoblot analysis (Fig. 1b), respectively. TACE expression in COS7 cells enhanced the release of sAPPα and the production of C83. Addition of NRDc to cells expressing TACE dramatically increased the production of sAPPα and C83, although sole expression of NRDc had a negligible effect on α-secretase's cleavage of APP. Similar results were obtained in

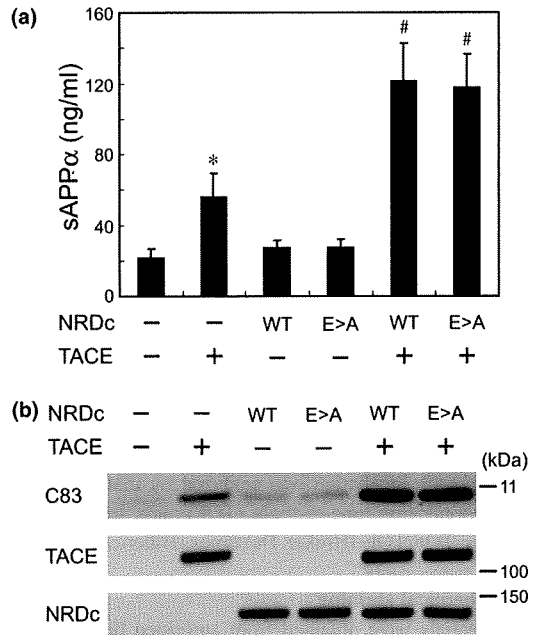

Fig. 1 Nardilysin (*N*-arginine dibasic convertase; NRDc) enhances the α-secretase activity of tumor necrosis factor-α converting enzyme (TACE). Human amyloid precursor protein (APP_{WT}) was transiently introduced into COS7 cells with the expression vector for NRDc (WT), an enzymatic inactive mutant of NRDc (E > A), and/or TACE as indicated. The culture medium of transfected cells was changed to a serum-free medium 24 h post-transfection. The conditioned medium and cell lysates were collected after an additional 4 h of incubation, as described under Materials and methods. The amounts of the soluble form of APP (sAPPα) in the conditioned medium and C-terminal fragment of APP (C-terminal fragment of 83 amino acids of APP; C83) in the cell lysates were determined by ELISA (a) and western blot analysis (b), respectively. Western blot analyses with anti-APP (C83), anti-V5 (NRDc), and anti-TACE were performed for cell lysates (b). The results are mean ± SD of three independent experiments. The asterisk (*), representing $p < 0.05$ (Student's *t*-test), indicates significant differences between control cells and TACE-transfected cells. The pound sign (#), representing $p < 0.01$, indicates significant differences between TACE-transfected cells and TACE/NRDc-transfected cells.

293T cells (data not shown). The expression levels of NRDc and TACE were comparable in COS7 cells transfected with both NRDc and TACE and cells transfected with only NRDc or TACE, indicating that NRDc and TACE had a synergistic effect on the cleavage of APP by α-secretase.

We also examined the effect of an enzymatically inactive mutant NRDc (Glu235 to Ala; E > A) (Nishi *et al.* 2006) on the α-secretase activity of TACE. Similar to the wildtype, the mutant NRDc and TACE coordinately enhanced the production of sAPPα and C83 (Fig. 1a and b), indicating that the metalloendopeptidase activity of NRDc is not required for the enhancement of TACE-induced α-secretase cleavage of APP. We have recently reported that the inactive NRDc, as well as

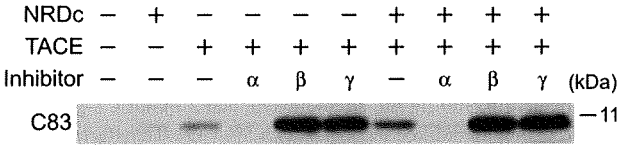

Fig. 2 Enhancement of α-secretase activity by Nardilysin (*N*-arginine dibasic convertase; NRDc) is totally abolished by a metalloprotease inhibitor, TAPI-2. Amyloid precursor protein (APP_{WT}) was transiently introduced into COS7 cells with the expression vector for NRDc and/or tumor necrosis factor-α converting enzyme (TACE) as indicated. After 24 h of transfection, the culture medium of transfected cells was changed to a serum-free medium with or without the metalloprotease inhibitor TAPI-2 (100 μmol/L), β-secretase inhibitor II (15 μmol/L), or γ-secretase inhibitor (70 μmol/L). After an additional 4 h of incubation, cell lysates were collected. Equivalent amounts of protein in each lane were analyzed by western blotting with anti-APP for the detection of C-terminal fragment of 83 amino acids of APP (C83).

wildtype NRDc, enhances TACE-induced HB-EGF shedding (Nishi *et al.* 2006), suggesting that a similar molecular mechanism is utilized for ectodomain shedding of two totally different membrane proteins, APP and HB-EGF.

Enhancement of α-secretase activity by NRDc is totally abolished by metalloprotease inhibitor

Hydroxamic acid-based zinc metalloprotease inhibitors have been shown to efficiently inhibit the cleavage of APP by α-secretase (Parvathy *et al.* 1998). We investigated the effect of the hydroxamic-based inhibitor TAPI-2 on α-secretase activity in COS7 cells over-expressing TACE with or without NRDc. TAPI-2 almost completely inhibited the production of C83 in cells expressing only TACE, as well as in cells expressing both NRDc and TACE (Fig. 2). We also examined the effects of β-secretase and γ-secretase inhibitors on the α-secretase activity. Both inhibitors dramatically elevated production levels of C83 (Fig. 2), which is consistent with reports that the amount of C83 increased in β-site APP-cleaving enzyme 1–/– mice (Luo *et al.* 2001) and in neuronal cells derived from presenilin-1–/– mice (De Strooper *et al.* 1998). These results indicate that NRDc-induced enhancement of the α-cleavage of APP occurs through the metalloprotease activity of TACE, which can be inhibited by TAPI-2.

Enhancement of TACE-induced peptide cleavage by NRDc

We have shown the direct interaction of NRDc with TACE (Nishi *et al.* 2006). To determine whether the direct binding of NRDc to TACE is sufficient for TACE's activation in the cleavage by α-secretase of APP, we performed an *in vitro* cleavage assay using an internally quenched fluorogenic peptide substrate containing the α-secretase cleavage site of APP. While TACE significantly increased the amount of fluorescence detected, addition of NRDc to TACE drama-

tically strengthened the fluorescence signal, indicating an enhancement of the cleavage by NRDc (Fig. 3a). Next, we performed the cleavage assay using $A\beta_{40}$ peptide and conducted a mass spectrometric analysis to confirm the cleavage site. The analysis showed that NRDc did not cleave the peptide at all, while TACE cleaved it between Lys16 and Leu17 of the Aβ sequence (Fig. 3b). The combination of NRDc and TACE also cleaved the peptide at the same site of the Aβ sequence (data not shown) indicating that NRDc potently activated the catalytic activity of TACE by direct binding.

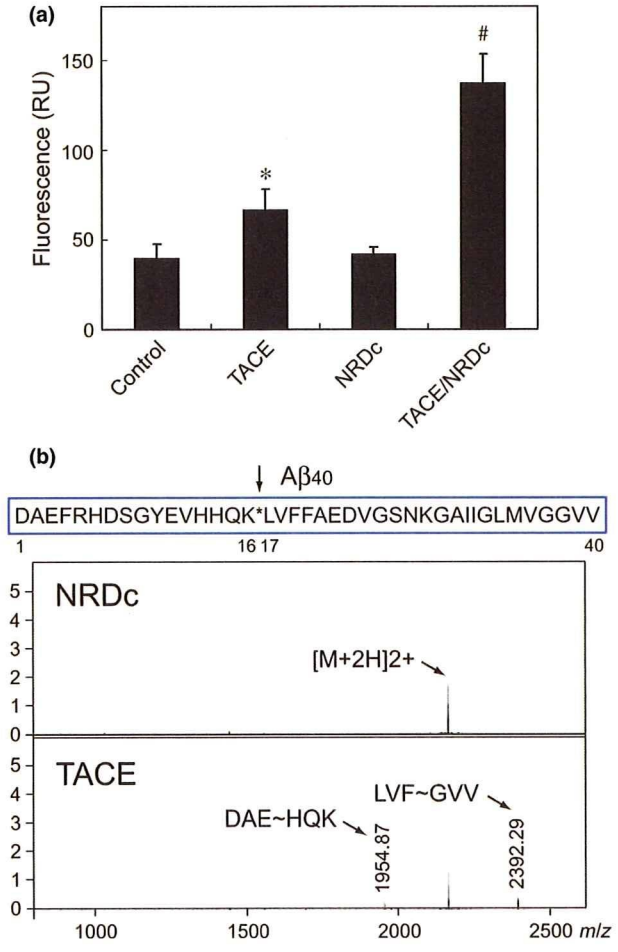

Fig. 3 Nardilysin (*N*-arginine dibasic convertase; NRDc) enhances tumor necrosis factor-α converting enzyme (TACE) -induced cleavage of amyloid β (Aβ) peptide. (a) A total of 100 μmol/L of the fluorescence-quenching peptide substrate containing the α-secretase cleavage site of amyloid precursor protein [Ac-RE(EDANS)-VHHQKLVF-K(DABCYL)-R-OH; Calbiochem] was incubated with combinations of recombinant TACE (5 μg/mL) and/or NRDc (5 μg/mL) as indicated. After 15 h of incubation at 37°C, fluorescence intensity (340/460 nm) was measured. The results are mean ± SD of four independent experiments. The asterisk (*), representing $p < 0.05$ (Student's *t*-test), indicates significant differences between the control and NRDc- or TACE-treatment. The pound sign (#), representing $p < 0.05$, indicates significant differences between TACE-treatment and TACE/NRDc-treatment. (b) A total of 100 μmol/L of $A\beta_{40}$ peptide was incubated with recombinant NRDc (25 μg/mL) or TACE (5 μg/mL). Samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. [M+2H]2+; doubly protonated molecule.

Enhancement of α-secretase activity of ADAM9 and ADAM10 by NRDc

ADAM9, ADAM10, and TACE are candidates for an α-secretase (Buxbaum *et al.* 1998; Koike *et al.* 1999; Lammich *et al.* 1999). To examine whether NRDc enhances the α-secretase activity of other ADAMs as well as TACE, we performed experiments in which NRDc was co-transfected with ADAM9, ADAM10, or TACE. Sole expression of any of the ADAM proteases increased the secretion of sAPPα (Fig. 4 upper panel) and production of C83 (Fig. 4a bottom panel) compared with cells expressing empty vector. Notably, the addition of NRDc to each ADAM significantly enhanced the production of sAPPα and C83 (Fig. 4a). These results indicate that NRDc can enhance the α-secretase activity of not only TACE but also several other ADAMs. To rule out non-specific enhancement of the cleavage of APP caused by protein over-expression, several unrelated proteins (LacZ, EGF receptor, and IQGAP-1) were over-expressed with TACE. As expected, none of these proteins affected the production of C83, indicating the specific effect of NRDc (Supplementary Fig. S1 for LacZ; data not shown for EGF receptor and IQGAP-1).

NRDc-mediated activation of α-secretase occurs through TACE and ADAM10

As shown in Figs 1, 2, 4a, and 5, sole expression of NRDc modestly increased sAPPα and C83 production, probably due to the activation of endogenous ADAM proteases. To clarify which of the ADAMs is involved in the NRDc-mediated activation of α-secretase, the effect of NRDc was examined in COS7 cells in which TACE was depleted by siRNA. The increase in the production of C83 by NRDc was dramatically reduced by gene knockdown of TACE (Fig. 4b). Depletion of ADAM10 also modestly decreased the enhancement of α-cleavage by NRDc (Fig. 4c). These results demonstrate that the enhancement of α-secretase activity by NRDc occurs specifically through ADAM proteases and that endogenous TACE and, to a lesser extent, ADAM10 are at least partially responsible for the NRDc-mediated modulation of α-secretase.

Activation of α-secretase by NRDc reduces Aβ generation

The APP_{SWE}, causative of familial AD, has a double mutation immediately flanking the N-terminus of the Aβ domain. When expressed in cells, the mutation causes the increased production of Aβ (Citron *et al.* 1992; Forman *et al.* 1997). Thus, APP_{SWE} instead of wild-type APP, is generally

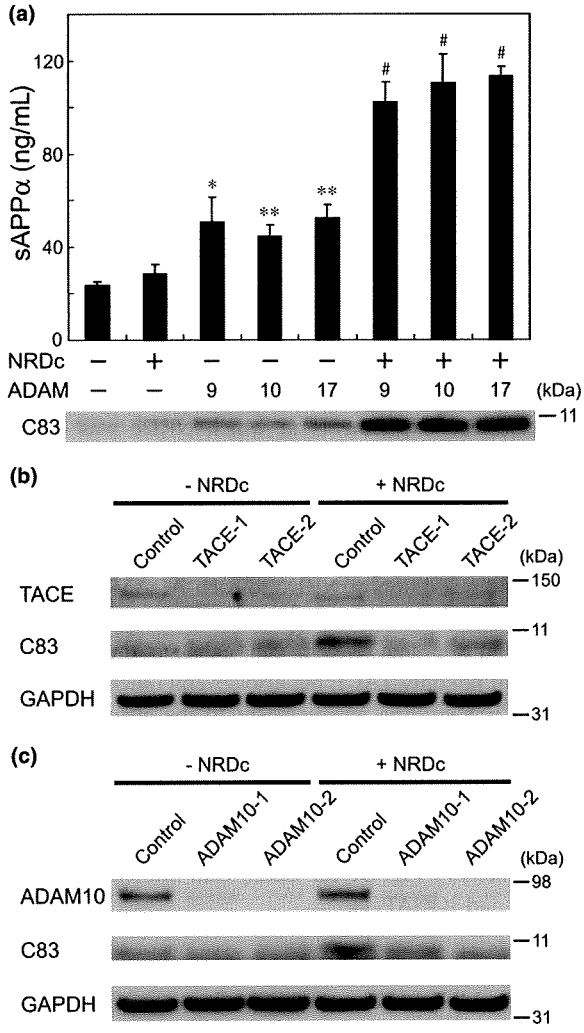

Fig. 4 (a) Enhancement of α-secretase activity of the ADAM9 (a disintegrin and metalloprotease 9) and ADAM10 by Nardilysin (*N*-arginine dibasic convertase; NRDc). COS7 cells were transiently transfected with the expression vector encoding ADAM9, ADAM10, and tumor necrosis factor-α converting enzyme (TACE)/ADAM17 in the presence or absence of the NRDc expression vector. Conditioned medium and cell lysates were collected as described in Fig. 1. The amounts of α-secretase-cleaved soluble APP (sAPPα) in the conditioned medium and C-terminal fragment of 83 amino acids of APP (C83) in the cell lysates were determined by ELISA (upper panel) and western blot analysis (bottom panel), respectively. The results are mean ± SD of three independent experiments. The asterisk (*), representing $p < 0.05$ (Student's *t*-test), and (**), representing $p < 0.01$, indicate significant differences between control cells and ADAM-trasfected cells. The pound sign (#), representing $p < 0.001$, indicates significant differences between ADAM-transfected cells and ADAM/NRDc-transfected cells. (b and c) Gene knockdown of ADAMs abrogates NRDc-mediated activation of α-secretase. COS7 cells were transiently co-transfected with APP_{WT} either with non-silencing control siRNA (control) or with siRNAs against TACE (TACE-1 and TACE-2) (b) or with those against ADAM10 (ADAM10-1 and ADAM10-2) (c). The culture medium was renewed after 24 h of incubation. After an additional 24 h of incubation, cell lysates were collected and expression levels of C83, glyceraldehyde-3 phosphate dehydrogenase (GAPDH), and TACE (b) or ADAM10 (c) were determined by western blot analysis.

used in cell-based transfection assays to detect Aβ (Forman *et al.* 1997). To examine whether the enhancement of α-secretase activity by NRDc is accompanied by a decrease in the amount of Aβ generated, we performed experiments in which NRDc and/or TACE were co-expressed with APP_{SWE} in COS7 cells. While the generation of Aβ was not affected by the sole expression of NRDc, it was reduced 20.5 ± 5.0% by the expression of TACE (Fig. 5b) concomitantly with increased secretion of sAPPα (Fig. 5a upper panel) and production of C83 (Fig. 5a bottom panel). The expression of NRDc together with TACE further reduced the amount of Aβ generated by 15.0 ± 4.2%, compared with the sole expression of TACE (Fig. 5b), indicating that NRDc is involved in regulating the production of Aβ.

Gene knockdown of NRDc results in decreased α-secretase activity and increased Aβ peptide generation

RNA-mediated interference of NRDc was used to confirm whether NRDc is essential for the cleavage of APP by α-secretase and generation of Aβ. We used mouse neuroblastoma N2a cells because (i) the endogenous level of NRDc is high enough to be detected by western blotting and (ii) N2a cells expressing APP_{WT} constitutively secrete sAPPα and Aβ in the conditioned medium at levels which can be detected by ELISA. Transfection of two different small interference RNA duplexes (siRNA) against mouse NRDc (mNRDc1 and mNRDc2) resulted in a reduction in the level of endogenous NRDc protein by 48.0 ± 8.1% (mNRDc1) and 53.2 ± 8.6% (mNRDc2), respectively, while neither siRNA affected the expression of full-length APP and glyceraldehyde-3 phosphate dehydrogenase (Fig. 6a). The amount of sAPPα released in the conditioned medium by α-secretase decreased by 31.0 ± 4.1% in mNRDc1 siRNA-transfected cells and by 58.2 ± 3.2% in mNRDc2 siRNA-transfected cells, compared with the cells transfected with control siRNA (Fig. 6b). On the other hand, the amount of $Aβ_{40}$ peptide released into the medium from mNRDc1 and mNRDc2 siRNA-transfected cells increased 141.5 ± 6.9% (mNRDc1) and 155.8 ± 7.1% (mNRDc2), compared with that released from control siRNA-transfected cells (Fig. 6c). These results further establish that NRDc plays an essential role in the cleavage by α-secretase of APP and production of Aβ.

NRDc is expressed in cortical neurons

To examine the expression of NRDc in the human brain, an immunohistochemical analysis was performed in autopsied brains of patients who died from non-neurological diseases.

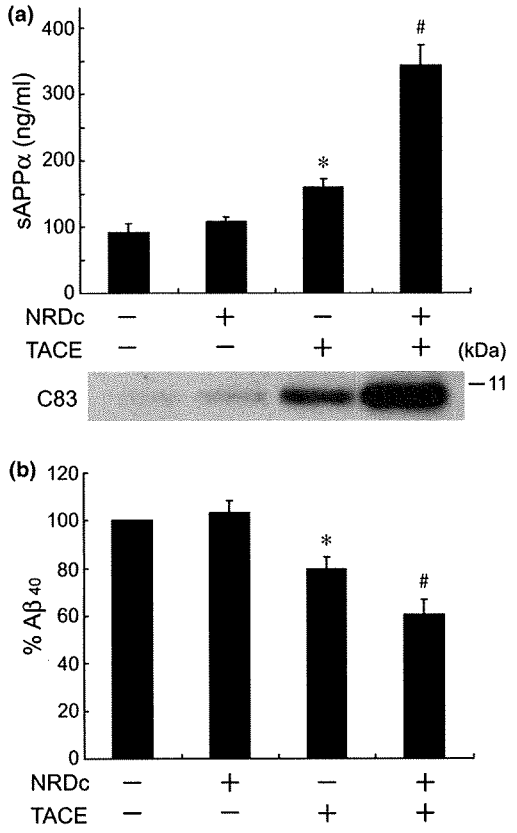

Fig. 5 Activation of α-secretase by Nardilysin (*N*-arginine dibasic convertase; NRDc) reduces the amount of amyloid β (Aβ) generated. Swedish mutant APP (APP_{SWE}) was transiently introduced into COS7 cells with the expression vector for NRDc and/or tumor necrosis factor-α converting enzyme (TACE) as indicated. Conditioned medium and cell lysates were collected as described in Fig. 1. The amounts of sAPPα ((a) upper panel) and Aβ (b) in the conditioned medium were determined by ELISA and C-terminal fragment of 83 amino acids of APP (C83) in the cell lysates was detected by western blot analysis ((a) bottom panel). The results are mean ± SD of four independent experiments. The asterisk (*), representing $p < 0.001$ (Student's *t*-test), indicates significant differences between control and TACE-transfected cells. The pound sign (#), representing $p < 0.005$, indicates significant differences between TACE-transfected and TACE/NRDc-transfected cells.

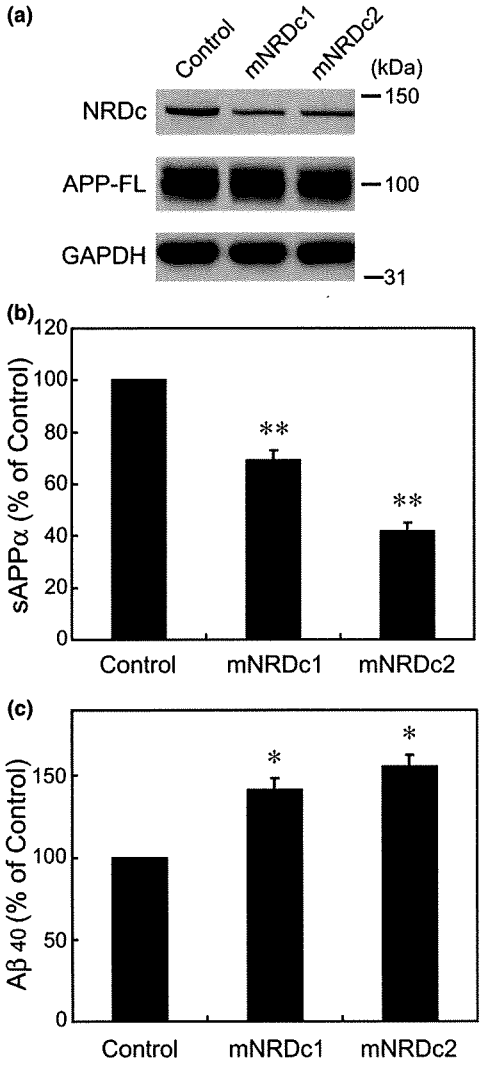

Fig. 6 Gene knockdown of Nardilysin (*N*-arginine dibasic convertase; NRDc) decreases α-secretase activity and increases amyloid β (Aβ) peptide generation. Neuro2a cells were transiently co-transfected with amyloid precursor protein (APP_{WT}) either with siRNA against mouse NRDc (mNRDc1 and mNRDc2) or non-silencing control siRNA (control). The culture medium was renewed after 24 h of incubation. After an additional 24 h of incubation, conditioned medium and cell lysates were collected. (a) Expression levels of NRDc, full-length APP (APP-FL), and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were determined by western blot analysis. The amounts of secreted sAPPα (b) and Aβ (c) in the conditioned medium were determined by ELISA as described in Materials and methods. The results are mean ± SD of five independent experiments. Statistical significance between control cells and siRNA-treated cells was determined with Student's *t*-test (*$p < 0.001$ and **$p < 0.0001$).

The specificity of the mouse monoclonal antibody against NRDc (#304) used for the staining was validated by two observations. First, immunocytochemical staining with #304 of COS7 cells transiently transfected with NRDc-V5 showed positive staining in cells expressing NRDc-V5, which were similarly stained with anti-V5 antibody (Fig. 7a). Antibody #304, but not anti-V5 antibody detected endogenous NRDc expressed in the perinuclear region. Second, western blotting with #304 of total cell lysates of COS7 cells expressing NRDc-V5 showed a single 140-kDa band (Fig. 7b, lane 2), which was also detected by anti-V5 antibody (Fig. 7b, lane 4). Endogenous NRDc of non-transfected cells was faintly detected by #304 (Fig. 7b, lane 1), but not detected by anti-V5 (Fig. 7b, lane 3). Serial sections of the frontal lobe were stained with silver impregnation (Fig. 7c) and anti-NRDc antibody #304 (Fig. 7d). A high-power view of the white

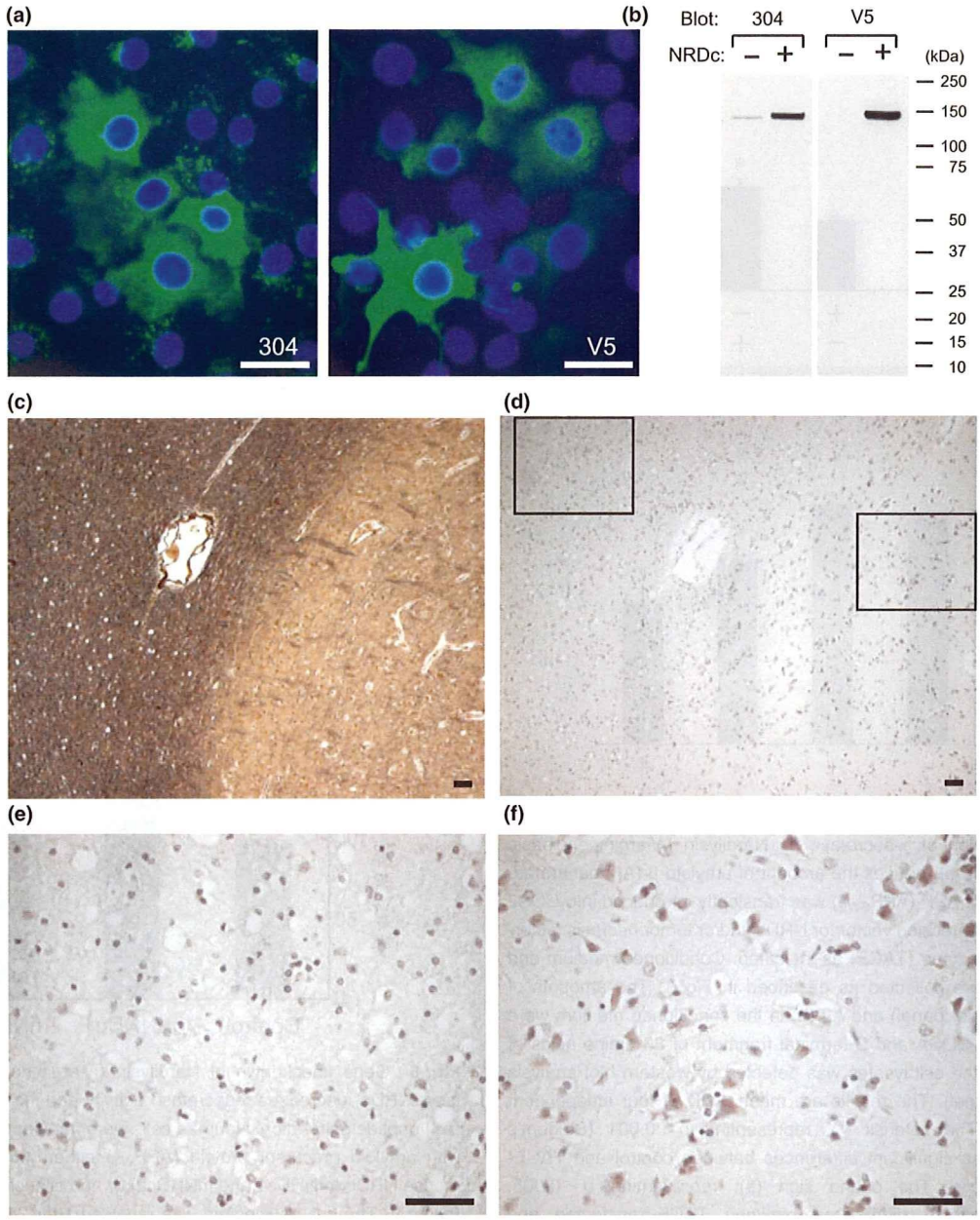

Fig. 7 Nardilysin (*N*-arginine dibasic convertase; NRDc) is expressed in cortical neurons of human brain. (a) COS7 cells were transiently transfected with the expression vector of NRDc-V5, fixed, and stained either with anti-NRDc antibody (#304: left panel) or with anti-V5 antibody (right panel). Alexa Fluor 488 donkey anti-mouse IgG was used as secondary antibody and the nucleus was subsequently stained with 4′,6-diamidino-2-phenylindole. Stained cells were visualized by fluorescence microscopy. Scale bar = 5 μm. (b) Total cell lysates of COS7 cells transiently transfected with control vector (lane 1 and 3) or expression vector of NRDc-V5 (lane 2 and 4) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was performed with anti-NRDc antibody (#304; lane 1 and 2) or anti-V5 antibody (V5; lane 3 and 4). (c and d) Serial sections of the frontal lobe were stained with silver impregnation (c) and anti-NPDc antibody (#304) (d). Areas in rectangles, one in the white matter (left) and the other in the cortex (right), are shown at a higher magnification in e and f. (e and f) Immunoreactivity of NRDc in the white matter (e) and the frontal cortex (f). Note that NRDc was strongly expressed in cortical neurons, while only weak immunoreactivity was detected in some glial cells in the cortex and the white matter. Scale bar = 5 μm in c through f.

matter (Fig. 7e) and the cortex (Fig. 7f) showed that NRDc is expressed almost exclusively in cortical neurons, particularly in large neurons. In contrast, NRDc expression was not apparent in glial cells. The obvious expression of NRDc in neurons further indicates a role for the metalloendopeptidase in the metabolism of Aβ.

Discussion

Our findings demonstrate that a metalloendopeptidase, NRDc, is involved in the metabolism of APP through the enhancement of α-secretase activity. In cells, NRDc enhanced the secretion of sAPPα and reduced the amount of Aβ peptide generated in cooperation with TACE. The enhancement of sAPPα's secretion by NRDc was abolished by TAPI-2, an efficient inhibitor for TACE, or siRNA-mediated gene knockdown of TACE, suggesting that the enhancement is due to TACE's activation. Furthermore, recombinant NRDc protein enhanced the TACE-induced cleavage of Aβ in the *in vitro* peptide cleavage assay, indicating that NRDc directly triggers the α-secretase activity of TACE. NRDc, a metalloendopeptidase, itself appears not to cleave APP because (i) a recombinant NRDc did not cleave Aβ peptide and (ii) an enzymatic inactive mutant of NRDc retained the ability to enhance the α-secretase activity of TACE. These results reinforced our previous finding that NRDc enhances the sheddase activity of TACE for HB-EGF, which was not dependent on the peptidase activity of NRDc (Nishi *et al.* 2006).

Originally, we identified NRDc as a specific-binding partner of HB-EGF (Nishi *et al.* 2001) and demonstrated that this metalloendopeptidase is a potent activator of the ectodomain shedding of HB-EGF (Nishi *et al.* 2006). In this study, we have clearly shown that the effect of NRDc is not specific to HB-EGF shedding. Given that NRDc enhanced the ectodomain shedding of two totally unrelated membrane protein substrates for TACE, HB-EGF, and APP, NRDc might be a general activator for the ADAM protease. Our pilot study showing that NRDc enhances ectodomain shedding of other EGF receptor ligands and TNF-α (Y. Hiraoka, K. Yoshida, M. Ohno, T. Kita and E. Nishi, manuscript in preparation), further supports this idea. An important question to be answered is whether the effect of NRDc on sheddases activation is limited to TACE or not. Other than TACE, ADAM9 and ADAM10 have been possible candidates for an α-secretase (Koike *et al.* 1999; Lammich *et al.* 1999). Co-transfection experiments clearly demonstrated that NRDc enhances the cleavage by α-secretase induced by these ADAM proteases. The findings suggest that NRDc could be an activator for at least several ADAM proteases, and thus an activator for ectodomain shedding of a broad spectrum of membrane proteins. Previously, we demonstrated the formation of a complex between NRDc and TACE in cells, which is promoted by phorbol esters (Nishi *et al.* 2006). Moreover, we demonstrated the direct binding of recombinant forms of NRDc and TACE, which resulted in the enhancement of TACE enzymatic activity (Nishi *et al.* 2006). Thus, we have concluded that the binding of NRDc to TACE at least partially explains how HB-EGF shedding is enhanced by phorbol ester, a general activator of ectodomain shedding. A similar molecular mechanism might exist for ADAM9 and ADAM10, enhancing their sheddase activity. However, further study of the interaction of NRDc with ADAM proteases is needed.

Our findings indicate that NRDc is required for α-cleavage of APP because RNAi-mediated inhibition of its endogenous expression in N2a cells was accompanied by a reduction in the α-cleavage of APP. Furthermore, the inhibition resulted in an increase in the amount of Aβ generated. Secretases of type α and β compete to cleave the ectodomain of APP (Vassar *et al.* 1999; Skovronsky *et al.* 2000), a process which seems to be regulated by certain mechanisms. For example, activation with phorbol esters favors α-cleavage even in cells expressing the APP_{SWE} form of APP, cells which otherwise show β-cleavage (Skovronsky *et al.* 2000). In this study, we have demonstrated that NRDc induces a shift from β-cleavage to α-cleavage for ectodomain shedding of APP. This observation indicated that NRDc might be a key regulatory element for the fate of APP ectodomain shedding and Aβ production.

Several experiments *in vitro* and *in vivo* have indicated that the ADAM proteases are candidates for α-secretases (Buxbaum *et al.* 1998; Koike *et al.* 1999; Lammich *et al.* 1999; Hooper and Turner 2002; Allinson *et al.* 2003; Postina *et al.* 2004). Postina *et al.* reported that neuronal over-expression of ADAM10 in mice promoted secretion of sAPPα, reduced the amount of Aβ peptide generated, and prevented amyloid plaques from forming (Postina *et al.* 2004). Their report is the first evidence that an ADAM protease acts as a α-secretase *in vivo*. Our findings revealed that co-expression of NRDc enhanced the α-secretase activity of ADAM9, ADAM10, and TACE in cells. Furthermore, we demonstrated a neuronal expression of NRDc in human brains. An intriguing question is whether NRDc enhances α-secretase activity and decreases Aβ production *in vivo*. To answer this question, we are now preparing transgenic mice over-expressing NRDc in neurons. Activation of α-secretase could be an alternative to the inhibition of β- or γ-secretase for the treatment of AD, because α-secretase cleaves APP within the Aβ sequence and thus precludes Aβ production. If the effect of NRDc on Aβ production can be confirmed in an animal model, a novel molecular mechanism of α-secretase's activation, involving the NRDc-ADAM protease system, could provide a new target for the treatment of AD.

NRDc belongs to the M16 family of metalloproteinases. Another member of this family, insulin-degrading enzyme (IDE), is the only mammalian protein having significant homology with NRDc (41% identity in the rat) (Affholter *et al.* 1988; Baumeister *et al.* 1993; Pierotti *et al.* 1994). IDE degrades Aβ, and there is compelling evidence for its role as an *in vivo* Aβ-degrading protease (Farris *et al.* 2003; Leissring *et al.* 2003). In spite of significant homology between IDE and NRDc, NRDc cannot degrade Aβ, as confirmed by mass spectrometry and western blotting (data

not shown). It is intriguing that two homologous members of the M16 family, IDE and NRDc, have inhibitory effects on the accumulation of Aβ via different mechanisms, degradation of Aβ and activation of α-secretase, respectively.

In conclusion, this study provides the first evidence that NRDc enhances cleavage by α-secretase through the activation of ADAM proteases, which results in a decrease in the amount of Aβ produced. More studies on the novel mechanisms by which α-secretase activity is regulated might lead to a new therapeutic approach to the treatment of AD.

Acknowledgements

We are grateful to Dr Y. Kaziro for his constant encouragement. We thank N. Nishimoto and H. Nakabayashi for excellent technical assistance; Drs A. Sehara, A. Kinoshita, and K. Matsumoto for providing materials. Financial support for this work was provided by grants from Special Coordination Funds for Promoting Science and Technology, Research Grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (18590809). This work was also supported by Grants-in-Aid for Young Scientists (18790305) and by grants from the Ichiro Kanehara Foundation.

Supplementary material

The following supplementary material is available for this article online:

Fig. S1 NRDc, but not LacZ, enhances the α-secretase activity of TACE.

This material is available as part of the online article from http://www.blackwell-synergy.com.

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