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Original Article

Apolipoprotein B-48 to Triglyceride Ratio Is a Novel and Useful Marker for Detection of Type III Hyperlipidemia after Antihyperlipidemic Intervention

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Aim: Remnant lipoproteins are atherogenic and are accumulated in patients with type III hyperlipidemia (HL). Although type III HL is diagnosed by phenotyping apolipoprotein (apo) E, this procedure is time-consuming and inconvenient for routine clinical use. Clinical indices for screening type III HL in untreated HL patients have been proposed; however, in clinical settings, HL patients are promptly treated with lipid-lowering agents without diagnosing the underlying cause. We investigated whether existing clinical indices for screening type III HL as well as the apo B-48/triglyceride (TG) ratio, which was suggested to be related to the accumulation of small chylomicron (CM) remnants, are useful after the initiation of lipid-lowering therapies.
Methods: In 25 normolipidemic subjects and 191 treated HL patients (type I, $n=6$; IIa, 62; IIb, 66; III, 12; IV, 22; and V, 23) from Osaka University Hospital and related hospitals, fasting low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), TG, and apolipoproteins were measured and clinical indices were evaluated statistically.
Results: Apo B-48 levels were significantly higher in patients with type I, III, and V HL, and TG levels were significantly higher in patients with type I and V HL. The apo B-48/TG ratio was significantly higher only in patients with type III HL compared with other types of HL ($p<0.001$), and was statistically significant among the other clinical indices (AUC-ROC value, 0.895; cut-off value, 0.110).
Conclusion: The apo B-48/TG ratio is a novel and useful marker for detecting type III HL even after the initiation of lipid-lowering interventions.

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Key words; Apolipoprotein B-48, Type III hyperlipidemia, Chylomicron remnants, Coronary heart disease

Introduction

Type III hyperlipidemia (HL) is a rare familial disorder characterized by a combined elevation in serum total cholesterol (TC) and triglycerides (TG), based on the marked accumulation of remnant lipoproteins, including chylomicron (CM) and VLDL remnants[1]. Many recent studies have proved that oxidized LDL and remnant lipoproteins have atherogenic features[2,3] and a strong correlation with the morbidity of coronary heart disease (CHD)[4-7]; therefore, type III HL should be diagnosed and treated as soon as possible.

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Type Ⅲ HL can be definitively diagnosed by phenotyping (genotyping) apo E (mainly apo E2/E2) using Western blotting or isoelectric focusing, and by demonstrating the presence of a broad β pattern (consisting of β-VLDL, IDL or VLDL remnants) on agarose gel electrophoresis or polyacrylamide gel disc electrophoresis (PAGE)[8-11]; however, these analyses are time-consuming and inconvenient for routine clinical use. Moreover, in clinical settings, HL patients are promptly treated with diet and exercise therapy as well as lipid-lowering agents without diagnosing the underlying cause.

Five groups of investigators have identified clinical indices for screening type Ⅲ HL. The apo E/apo B ratio was significantly greater in patients with type Ⅲ HL than in those with other types of HL[12]. The apo B/TC ratio was significantly lower in patients with dysbetalipoproteinemia whose apo E genotype was E2/E2 than in those with mixed HL[13]. A simple algorithm was developed for the diagnosis of type Ⅲ HL on the basis of a TC/apo B ratio of >6.2 and a TG/apo B ratio of <10.0, which were not detected in patients with other types of HL[14]. The non-HDL-C/apo B ratio was significantly greater in patients with type Ⅲ HL and an apo E genotype of E2/E2 than in those with combined HL other than type Ⅲ HL or hypothyroidism[15]. In Japanese patients with type Ⅲ HL (apo E2/2 phenotype), the apo E/C-Ⅲ index was considerably higher than in patients with other types of HL[16]. As these studies were well designed and had high sensitivity and specificity for screening untreated type Ⅲ HL, these clinical criteria might be distorted and their specificity and sensitivity might decrease after lipid-lowering intervention. Drug withdrawal for the diagnosis of type Ⅲ HL may increase the risk of cardiovascular events; therefore, practical and simple criteria for screening type Ⅲ HL after lipid-lowering intervention must be established.

A novel sandwich enzyme-linked immunosorbent assay (ELISA)[17] and chemiluminescent enzyme immunoassay (CLEIA) system[18] to measure serum apo B-48 were developed. Fasting apo B-48 levels are correlated with postprandial TG increase[19] and are significantly higher in patients with accumulated CM and CM remnants[17] and metabolic syndrome (MetS)[20]. High fasting apo B-48 levels are correlated with premature carotid artery stenosis[21, 22], and postprandial increases in TG and apo B-48 are correlated with CHD[23]. Emerging evidence has suggested that CM remnants are responsible for the initiation and development of atherosclerotic plaques *in vitro* and *in vivo*[2]. In our previous study, we observed high fasting serum TG and apo B-48 in patients with type Ⅲ HL and the apo B-48 to TG (B-48/TG) ratio was significantly higher only in patients with type Ⅲ HL than in those with other types of HL, none of whom had received any lipid-lowering interventions[17]. It has been suggested that a high apo B-48/TG ratio is related to the accumulation of small CM remnants, which contain one molecule of apo B-48 and smaller amounts of TG than those of the larger CMs or CM remnants. Small CM remnants may be accumulated after therapy because of a genetic disorder of apoE, and a high apoB-48/TG ratio might be useful for detecting type Ⅲ HL even after the initiation of lipid-lowering interventions.

Thus, the aim of the present study was to evaluate whether the apo B-48/TG ratio is useful for detecting type Ⅲ HL even after the initiation of lipid-lowering interventions.

Subjects and Methods

Subjects and Patients

Subjects with NL and patients with HL were identified from candidates registered in the central registration database for evaluating the clinical usefulness of apo B-48 in patients with dyslipidemia, diabetes mellitus, MetS, and CHD. These candidates consisted of cardiovascular medicine outpatients at Osaka University Hospital (n=151) and another university hospital in Japan (n=65). Fasting TC and TG levels at the time of diagnosis, before any lipid-lowering interventions for dyslipidemia were administered, were examined for all subjects (n=216). HL was diagnosed using criteria based on the Japan Atherosclerosis Society (JAS) guidelines for the diagnosis and prevention of atherosclerotic cardiovascular diseases among Japanese individuals[24]. First, 25 subjects were considered to have NL as they had low TC and TG levels (TC <220 mg/dL and TG <150 mg/dL). The remaining 192 subjects were diagnosed with HL as they had high TC and TG levels (TC ≥220 mg/dL and/or TG ≥150 mg/dL) and had not received any lipid-lowering agents. The HL phenotype was determined based on the criteria proposed by the Research Committee for Primary Hyperlipidemia of the Ministry of Health and Welfare of Japan[24]. The diagnosis of type Ⅲ HL (n=12) with the apo E2/2 phenotype was performed based on the standard criteria previously reported[25]. All patients with HL were already undergoing treatment with oral agents for hyperlipidemia, including statins, fibrates, probucol, nicotinic acid, and anion-exchange resins. This study was approved by the Ethics Committee of Osaka University Hospital and the other university hospital; informed consent was obtained from all participants in this study.

Table 1. Clinical and biochemical profiles of patients with hyperlipidemia

	NL	Type I	Type IIa	Type IIb	Type III	Type IV	Type V
Male/Female	12/13	4/2	44/18	31/35	5/7	6/16	3/20
Medications							
Statin (%)		0	67.7	54.5	8.3	27.3	13.0
Fibrate (%)		33.3	18.2	18.2	100	63.6	39.1
Probucol (%)		0	3.0	3.0	0	9.1	0
Nicotinic acid (%)		16.7	6.1	6.1	0	13.6	4.3
Colestimide (%)		0	1.5	1.5	0	0	0
Ezetimibe (%)		0	6.1	6.1	0	0	0
Age (years)	67±15	44±20***	66±10	64±10	66±15	64±9	54±13***
BMI (kg/m^2)	24.0±4.6	23.4±5.0	23.3±3.6	24.0±3.6	25.4±2.9	24.5±3.9	25.4±3.8
BP (mmHg)							
Systolic BP	144±18	114±13*	139±22	139±12	139±22	126±18	126±10*
Diastolic BP	84±13	69±8	81±12	79±12	80±11	82±9	79±10
TC (mg/dL)	195±31	227±59	226±49*	210±47	209±63	192±34	237±65*
HDL-C (mg/dL)	59±14	31±14***	67±16*	56±13	60±12	43±15***	41±11***
LDL-C (mg/dL)	118±23	76±40	137±44	122±25	85±47	115±31	111±47
TG (mg/dL)	95±44	1096±610***	103±62	156±71	213±122	190±120	571±440***
ApoB (mg/dL)	91±19	79±34	107±27	107±25	84±48	105±24	120±32**
ApoE (mg/dL)	4.9±1.1	10.5±2.8***	4.9±1.5	4.9±1.3	10.3±3.4***	6.3±4.9	8.6±4.9***
ApoCIII (mg/dL)	8.2±2.4	21.9±5.3***	10.3±3.0	11.1±4.0	13.4±5.4	12.1±8.1	20.1±12.8***
nonHDL-C (mg/dL)	136±27	195±63*	159±49	144±28	148±67	142±47	186±76***
ApoB48 (μg/mL)							
Median	2.6	21.4**	2.5	3.2	21.2***	3.0	16.2***
(25-75th percentile)	(2.0-3.6)	(18.1-33.6)	(1.7-4.1)	(2.3-6.6)	(11.6-33.9)	(1.8-9.8)	(7.4-28.2)
ApoB48/TG ratio							
Median	0.032§	0.026§	0.029§	0.026§	0.126***	0.022§	0.036§
(25-75th percentile)	(0.021-0.045)	(0.016-0.035)	(0.020-0.040)	(0.019-0.037)	(0.055-0.194)	(0.151-0.030)	(0.021-0.053)

All data, except for nonparametric variables (apoB-48 and apoB-48/TG ratio), are expressed as the means ± S.D. or frequencies . ApoB-48 and apoB-48/TG ratio are expressed as medians, 25th percentile and 75th percentile. Normally distributed variables were analyzed by one-way ANOVA with Dunnett's multiple comparison test and the nonparametric variables (apoB-48 and apoB-48/TG ratio) were analyzed by the Kruskal-Wallis test with Steel's test between NL and other types of HL. *$p<0.05$ vs NL, **$p<0.01$ vs NL, ***$p<0.001$ vs NL. Differences of the apoB-48/TG ratio between patients with type III HL and subjects with NL or other types of HL were analyzed using Wilcoxon's rank sum test. §$p<0.001$ vs type III HL.

Measurements

In all subjects, height and weight were measured, body mass index (BMI) was calculated, and blood pressure was measured in a sitting position in the morning. Blood samples were collected in the morning after an overnight fast. Sera were separated immediately by low-speed centrifugation (3,000×g) for 10 min at 4℃ and stored at −80℃ until used. Serum TC and TG were determined by an enzymatic method; serum LDL-C and HDL-C levels by a direct method using Cholestest N HDL (Sekisui Medical Co Ltd, Tokyo, Japan) and Cholestest LDL (Sekisui Medical Co Ltd, Tokyo, Japan); and serum apo B, apo E, and apo C-III levels by an immunoturbidity method (Sekisui Medical Co Ltd). Serum apo B-48 levels were determined using our own CLEIA system (Fuji Rebio Inc., Tokyo, Japan)[18]. A broad β pattern of serum lipoproteins was determined by PAGE, and the presence of the apo E phenotype was determined by isoelectric focusing gel electrophoresis (JOKOH Co., Tokyo, Japan)[26]. All samples were treated in accordance with the Helsinki Declaration[27]. Non-HDL-C levels were calculated by subtracting HDL-C values from TC values. Clinical indices for screening type III HL (apo E/apo B ratio, apo B/ TC ratio, TC/apo B ratio, TG/apo B ratio, non-HDL-C/apo B ratio, and apo E/C-III ratio) were calculated[12-16] in addition to the apo B-48/TG ratio[17].

Statistical Analysis

Biochemical parameters concerning lipid and lipoprotein profiles without apo B-48 were normally

Table 2. Clinical and lipid profiles in patients with type Ⅲ hyperlipidemia

Case	Gender	Age (years)	ApoE phenotype	Lipid-lowering drug	TC (mg/dL)	TG (mg/dL)	ApoB-48 (μg/mL)	ApoB (mg/dL)	ApoE (mg/dL)
1	M	65	E2/E2	Fenofibrate	155	254	8.9	49	8.8
2	M	59	E2/E2	Fenofibrate	193	120	13.2	59	10.9
3	M	78	E2/E2	Bezafibrate	173	221	25.7	66	9.8
4	F	80	E2/E2	Bezafibrate	194	123	16.7	61	9.0
5	F	64	E2/E2	Bezafibrate	216	195	32.5	86	13.6
6	F	85	E2/E2	Fenofibrate	138	62	11.1	38	7.3
7	M	82	E2/E2	Fenofibrate	232	208	41.2	87	11.5
8	M	66	E2/E2	Fenofibrate + Pravastatin	224	179	37.8	81	17.8
9	M	45	E2/E2	Fenofibrate	245	286	29.8	86	12.8
10	M	55	E2/E2	Bezafibrate	235	268	10.0	124	5.1
11	M	36	E2/E2	Bezafibrate	372	581	13.2	216	8.5
12	F	75	E2/E2	Bezafibrate	145	152	34.4	52	9.2

Case	ApoCⅢ (mg/dL)	non-HDL-C (mg/dL)	ApoB48/TG	ApoE/apoB	apoB//TC	TC/apoB	TG/apoB	non-HDL-C/ apoB	apoE/ apoCⅢ
1	0.9	104	0.035	0.18	0.32	3.16	5.18	2.12	0.88
2	14.8	113	0.110	0.18	0.31	3.26	2.03	1.91	0.74
3	9.3	120	0.116	0.15	0.38	2.62	3.35	1.82	1.05
4	10.3	121	0.135	0.15	0.31	3.19	2.02	1.99	0.87
5	9.3	168	0.167	0.16	0.40	2.51	2.26	1.95	1.46
6	3.8	59	0.179	0.17	0.28	3.63	1.63	1.55	0.89
7	13.2	170	0.198	0.13	0.37	2.67	2.39	1.97	0.87
8	9.9	167	0.211	0.22	0.36	2.76	2.21	2.06	1.80
9	19.0	175	0.104	0.15	0.35	2.84	3.32	2.03	0.67
10	21.6	166	0.039	0.04	0.55	1.83	2.08	1.34	0.24
11	24.1	323	0.025	0.04	0.58	1.72	2.48	1.50	0.35
12	12.0	92	0.293	0.18	0.39	2.60	2.25	1.77	0.77

distributed variables, and apo B-48 levels were non-parametric variables[17, 21]; these levels are expressed as the mean ± SD. The overall difference in biochemical parameters, apoB-48 and the apoB-48/TG ratio was compared between subjects with NL and patients with any type of HL, one-way ANOVA with Dunnett's multiple comparison tests were used for the difference in biochemical parameters and the Kruskal-Wallis and Steel's tests were used for that in apo B-48 and the apo B-48/TG ratio. Moreover, differences in the apo B-48/TG ratio between patients with type Ⅲ HL and subjects with NL as well as patients with other types of HL were analyzed by Wilcoxon's rank sum test. Multiple comparisons in clinical indices for screening type Ⅲ HL[12-16] between subjects with NL and patients with different types of HL were analyzed by Dunnett's multiple comparison test or Steel's test (apo B-48/TG ratio) and those between patients with type Ⅲ HL and other types of HL were also analyzed by Dunnett's multiple comparison test or Steel's test. Statistical significance was declared if the one-sided p value was <0.05. Receiver-operating characteristic (ROC) curves were used to examine the value of the apo B-48/TG ratio useful for categorizing subjects on the basis of the presence of type Ⅲ HL. Data were analyzed by linear discriminant analysis, and the error rate for subjects with NL and subjects with other types of HL was evaluated relative to patients with type Ⅲ HL. Statistical analyses were performed using JMP 9 software (SAS Institute, Cary, NC).

Results

Clinical Profiles of Patients with HL after Anti-Hyperlipidemic Intervention

The clinical profiles and biochemical markers of lipid and lipoprotein metabolism were compared among subjects with NL and patients with type I, Ⅱa, Ⅱb, Ⅲ, Ⅳ, and Ⅴ HL (**Table 1**). Patients with HL were already receiving treatment with lipid-lowering drugs.

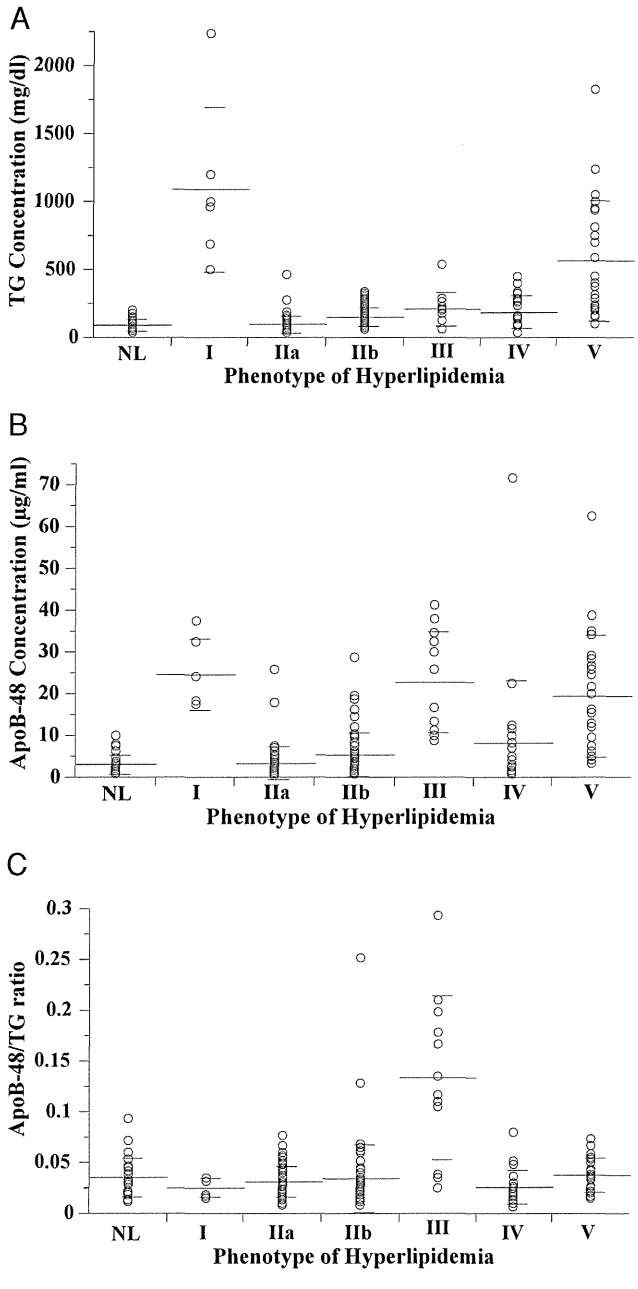

Fig. 1. Fasting serum apo B-48 levels in various types of hyperlipidemia after antihyperlipidemic treatments.

Fasting TG levels (A), apo B-48 levels (B) and apo B-48/TG ratio (C) were determined for 25 subjects with NL, and type I (n=6), type IIa (n=62), type IIb (n=66), type III (n=12), type IV (n=22), and type V (n=23) HL patients. Data are expressed as a scatter plot using the mean ± SD.

Patients with type I HL were treated with a low-fat diet, fibrates and nicotinic acids. Patients with type IIa and IIb HL were mainly treated with statins, and patients with type III, IV, and V HL were mainly treated with fibrates (**Table 1**). Clinical profiles of 12

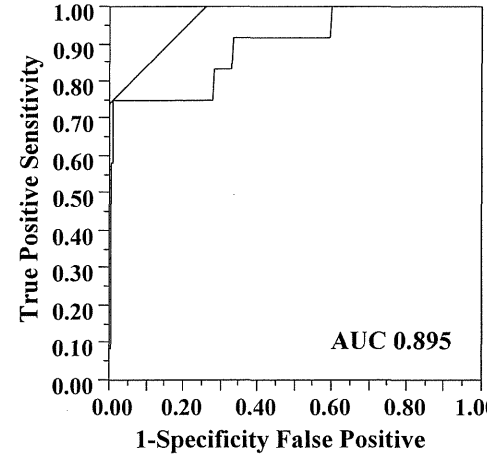

Fig. 2. ROC curve for Apo B48/TG ratio.

The receiver-operating characteristic (ROC) curve illustrating the utility of the apo B48/TG ratio in the diagnosis of type III HL. AUC: area under the curve.

patients with type III HL are shown in **Table 2**; these patients had already been treated with fibrates (fenofibrate or bezafibrate) and statins. TC levels were significantly higher among patients with type IIa and V HL than among subjects with NL after lipid-lowering treatments; however, there was no significant difference in LDL-C levels between these groups. TG levels were much higher among patients with type I and V HL than in subjects with NL, but not among patients with type III HL (**Table 1**, **2** and **Fig. 1**). Apo B levels were significantly higher among patients with type V HL than among subjects with NL, but not among patients with type IIa or IIb HL. Apo E levels were significantly higher among patients with type I, III, and V HL, and apo C-III levels were significantly higher among patients with type I and V HL than subjects with NL (**Table 1**).

ApoB-48 Levels and Apo B-48/TG Ratios

As shown in **Table 1** and **Fig. 1**, there was no significant difference in TG levels between patients with type III HL and subjects with NL; however, apo B-48 levels among patients with type I, III, and V HL were significantly higher than among subjects with NL ($p<0.001$) (**Table 1** and **Fig. 1**). The apo B-48/TG ratio was also significantly higher among patients with type III HL than among subjects with NL ($p<0.001$, assessed by Steel's multiple comparisons test) and patients with other types of HL ($p<0.001$, assessed by Wilcoxon's rank sum test). When discriminating patients with type III HL from patients with other types of HL, the apo B48/TG ratio yielded an AUC-ROC value of 0.895 (**Fig. 2**), indicating that the apo B-48/TG ratio maintained high accuracy for detecting

Table 3. Clinical indexes for screening type Ⅲ hyperlipidemia

	NL (n=25)	type Ⅰ (n=6)	type Ⅱa (n=62)	type Ⅱb (n=66)	type Ⅲ (n=12)	type Ⅳ (n=22)	type Ⅴ (n=23)
apoE/apoB[12]	0.05±0.01†	0.15±0.07***	0.05±0.02†	0.05±0.01†	0.15±0.05***	0.06±0.04†	0.07±0.04†
apoB/TC[13]	0.47±0.06†	0.37±0.16**	0.47±0.06	0.51±0.05*,†	0.38±0.09***	0.55±0.07***,†	0.51±0.06†
TC/apoB[14]	2.17±0.27§	3.75±3.17***,§	2.15±0.27§	1.88±0.45†	2.76±0.57*	1.78±0.46†	1.89±0.48†
TG/apoB[14]	1.02±0.36	20.99±24.3***,†	0.97±0.48	1.42±0.56	2.49±0.94	1.75±1.19	5.17±4.66**
non-HDL-C/apoB[15]	1.49±0.07	3.33±3.07***,†	1.48±0.11	1.03±0.64*,§	1.83±0.25	1.36±0.32	1.55±0.47
apoE/apoCⅢ[16]	0.30±0.04†	0.36±0.00†	0.40±0.03***,†	0.52±0.04***,†	0.62±0.01***	0.70±0.04***	0.97±0.25***
apoB48/TG ratio	0.036±0.019†	0.026±0.010†	0.032±0.016†	0.035±0.034†	0.137±0.08***	0.026±0.017†	0.038±0.017†
Median	0.032†	0.026†	0.029†	0.026†	0.126***	0.022†	0.036†
(25-75th percentile)	(0.021-0.045)	(0.016-0.035)	(0.020-0.040)	(0.019-0.037)	(0.055-0.194)	(0.151-0.030)	(0.021-0.053)

All data are expressed as the means±S.D. In addition, apoB-48/TG ratios are expressed as medians, 25th percentile and 75th percentile. Multiple comparisons between NL and any types of HL were analyzed by Dunnett's multiple comparison test or Steel's test (apoB-48/TG ratio). $^{*}p<0.05$ vs NL, $^{**}p<0.01$ vs NL, $^{***}p<0.001$ vs NL.
Multiple comparisons between type Ⅲ HL and NL or any types of HL were analyzed by Dunnett's multiple comparison test or Steel's test (apoB-48/TG ratio). $^{\S}p<0.05$ vs type Ⅲ HL, $^{\dagger}p<0.001$ vs type Ⅲ HL

type Ⅲ HL even after lipid-lowering interventions. The cut-off value of the apo B48/TG ratio was identified as 0.110; linear discriminant analysis revealed a 2.8% error rate for all types of HL and NL vs. type Ⅲ HL.

Apo B48/TG Ratio and Other Proposed Clinical Indices for Screening of Type Ⅲ HL

Many researchers have proposed clinical indices for screening type Ⅲ HL[12-16]; we evaluated whether these clinical indices and the apo B-48/TG ratio remained useful for screening type Ⅲ HL after the initiation of lipid-lowering interventions (**Table 3**). Analyses were performed with Dunnett's or Steel's multiple comparison tests; statistical significance of differences was observed between patients with type Ⅲ HL and those with other types of HL when we adopted the apo E/apo B, apo B/TC, TC/apo B, apo E/apo C-Ⅲ, and apo B-48/TG ratios. However, when the apo E/apo B, and TC/apo B ratios were adopted, a statistically significant difference was also observed between patients with type Ⅰ HL and those with other types of HL. When the apo B/TC ratio was adopted, a statistically significant difference was also observed between patients with type Ⅰ, Ⅱa, Ⅲ, and Ⅳ HL and those with other types of HL. Only when the apo B-48/TG ratio was adopted was a statistically significant difference observed only between patients with type Ⅲ HL and those with other types of HL.

Discussion

The accumulation of "atherogenic" remnants in patients with type Ⅲ HL is strongly correlated with the development of atherosclerotic cardiovascular diseases[6,7]. Nutritional or pharmacological interventions are administrated to these patients as soon as possible without investigating the etiology of dyslipidemia, so the assessment of HL type in patients with dyslipidemia on the basis of their biochemical data, such as TC and TG levels, is very difficult. In this study, we assessed a simple marker for screening type Ⅲ HL,the apoB-48/TG ratio, which is suitable for use even after initiation of lipid-lowering interventions.

Clinical Profiles of Type Ⅲ HL after Initiation of Lipid-Lowering Interventions

Appropriate intervention with lipid-lowering drugs reduces levels of lipids and apolipoproteins to within normal limits in some patients with HL (**Table 1**). TG and apo B-48 levels were decreased after administration of lipid-lowering drugs to patients with type Ⅰ, Ⅲ, Ⅳ, and Ⅴ HL compared with those before administration of these drugs, but not in patients with type Ⅱa and Ⅱb HL (17 and **Table 1**). Fibrate administration decreased TG levels in patients with type Ⅲ HL to the levels observed in subjects with NL, but it did not achieve a sufficient TG decrease in patients with type Ⅰ and Ⅴ HL (**Fig. 1A**). On the other hand, apo B-48 levels remained significantly high in patients with type Ⅲ HL as well as type Ⅰ and Ⅴ HL after the administration of fibrates. We suspected that these differences in lipid-lowering effects between type Ⅰ, Ⅲ, and Ⅴ HL may be due to increased deterioration of LPL activity by fibrates or nicotinic acids. Since fibrates enhance LPL-mediated catabolism of VLDL-TG and CM-TG, the TG content of remnant lipoproteins might have decreased and their particle sizes diminished. In patients with type Ⅲ HL, increased

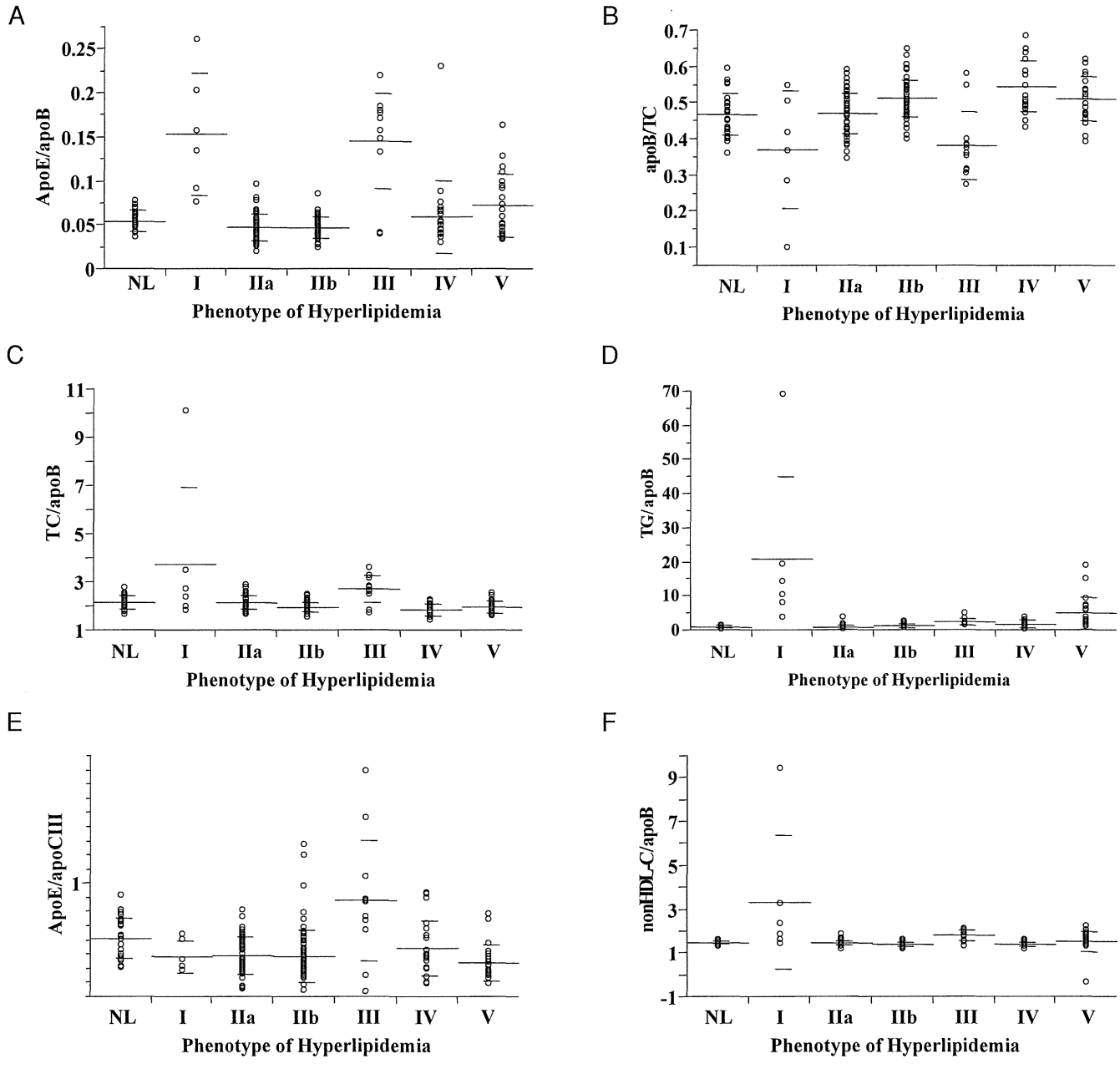

Fig. 3. Clinical indexes for screening type Ⅲ hyperlipidemia.

The apo E/apo B (A), apo B/TC (B), TC/apo B (C), TG/apo B (D), non HDL-C/apo B (E) and apo E/apo C-Ⅲ ratios (F) were determined in 25 subjects with NL, and type I (n=6), type Ⅱa (n=62), type Ⅱb (n=66), type Ⅲ (n=12), type Ⅳ (n=22), and type V (n=23) HL patients. Data are expressed as a scatter plot using the mean ± SD.

LPL activity may decrease VLDL-TG and CM-TG but not the number of VLDL and CM remnants, because liver uptake of them is impaired due to genetic apo E abnormality. Apo E and apo CⅢ were significantly higher in patients with type I, Ⅲ, and V HL (**Table 1**), which might also be due to impaired liver uptake of remnant lipoproteins. LPL activity is genetically impaired in patients with type I HL, and fibrates might not be able to decrease TG and apoB-48 levels. As a result, high apo B-48 levels and low TG levels were observed in patients with type Ⅲ HL after the administration of fibrates.

Clinical Indices for Screening Type Ⅲ HL after Lipid-Lowering Interventions

Five groups of investigators have developed and evaluated clinical indices for screening type Ⅲ HL before the initiation of lipid-lowering interventions[12-16]. We evaluated whether their clinical indices including the apoB-48/TG ratio were also useful for screening type Ⅲ HL when lipid and lipoprotein profiles were already improved by lipid-lowering agents (**Table 3** and **Fig. 3**). A high apo E/apo B ratio was based on the concept that the accumulation of remnant lipoproteins increases apo E but not apo B levels[12]; fibrates reduce apo B levels in patients with type I HL resulting in a high apo E/apo B ratio in patients with type I HL as well as type Ⅲ HL. A low apo B/TC ratio[13] was based on the concept that remnant lipoproteins are larger and contain more lipids than LDL particles; this increases serum cholesterol levels and decreases apo B levels. Since statins and fibrates effectively decrease both TC and apo B levels, low apo B/TC levels are observed in patients with type I HL as well as type Ⅲ HL (**Table 1**). In an algorithm by Sniderman *et al.*, a high TC/apo B ratio discriminated patients with type I, Ⅲ, and V HL, and a low TG/apo B ratio discriminated patients with type Ⅲ HL among them[14]. This might be based on the concept that the accumulation of remnant lipoproteins results in greater cholesterol content than that of LDL but a lower TG content than that of CM or VLDL. Statins and fibrates effectively decrease both TC and apo B levels, high TC/apo B levels were also observed in patients with type I as well as type Ⅲ HL and the TG/apo B ratio was significantly lower in patients with type Ⅲ HL than in those with type I HL (**Table 1**). This algorithm may be useful even after the administration of lipid-lowering agents; however, the cut-off value that divided type I or Ⅲ HL from the other types of HL was lower than that of the TC/apo B ratio reported by Sniderman *et al.* (TC/apo B >6.2)[14]. The non-HDL-C/apo B ratio relates to the cholesterol content in one particle of remnant lipoproteins and LDL, and a high non-HDL-C/apo B ratio indicates the accumulation of remnant lipoprotein and LDL[15]. Statins and fibrates decrease the cholesterol content of remnant lipoproteins in patients with type Ⅲ HL, and the non-HDL-C/apo B ratio was significantly highest in patients with not only type Ⅲ HL but also type I HL (**Table 3** and **Fig. 3**). The high non-HDL-C/apo B ratio in patients with type I HL may be caused by impaired LPL activity, which could not decrease CM and CM remnants even after lipid-lowering intervention with fibrates. A high apo E/apo C-Ⅲ ratio was based on the lipoprotein profile in which apo E levels were 3-fold higher but apo C-Ⅲ levels were 2-fold higher than in NL subjects, which might be due to the marked accumulation of remnant lipoproteins associated with apo E[16]. After lipid-lowering intervention, high apo E levels were still observed in patients with type I, Ⅲ, and V HL, and high apo C-Ⅲ levels were observed in patients with type I and V HL (**Table 1**); however, high apo E/apo C-Ⅲ ratios were observed in patients with not only type Ⅲ but also type Ⅱb, Ⅳ, and V HL (**Table 3** and **Fig. 3**). Both statins and fibrates significantly decreased VLDL-apo C-Ⅲ levels in patients with dyslipidemia by reducing the production and increasing the fractional catabolic rate of VLDL-apo C-Ⅲ[28, 29].

The apo B-48/TG ratio was the only index that could distinguish patients with type Ⅲ HL from those with other types of HL after the initiation of lipid-lowering interventions (**Table 1** and **3**). Fibrates strongly decrease TG levels in patients with accumulated TG-rich lipoproteins by increasing LPL activity; however, they only minimize the size of remnant lipoproteins and cannot improve hepatic uptake of these remnants because of the genetic dysfunction of apo E2/E2. We estimated that the apo B48/TG ratio yields an AUC-ROC value of 0.895 (**Fig. 2**), which indicates that the ratio has a high accuracy rate in detecting type Ⅲ HL (cut-off value, 0.110; error rate vs. type Ⅲ HL, 2.8%). Using an automated chemical analyzer in our CLEIA system (the results are available within 2 h), the apo B-48/TG ratio will provide a simple index for screening type Ⅲ HL before as well as after the administration of lipid-lowering agents.

Atherogenicity of Type Ⅲ HL after Lipid-Lowering Intervention

Emerging evidence has shown that CM remnants might be responsible for the initiation and development of atherosclerotic plaques *in vitro* and *in vivo*[2]. High fasting apo B-48 is correlated with premature carotid artery stenosis[21, 22] and the existence of CHD among other metabolic biomarkers related to coronary risk, and the combination with other impaired biomarkers represents a stronger risk state for CHD (Masuda D *et al.*, unpublished observation, 2011). Many *in vitro* and *in vivo* studies have shown that small CM remnants may play an important role in the initiation and development of atherosclerotic plaques[2]. The size of CMs and CM remnant particles produced from the intestine during the postprandial period has varied from a large CM to small LDL or HDL[2, 30]. A high apo B-48/TG ratio indicates the accumulation of small CM remnants that contain one apo B-48 molecule and lower TG content than in large CM particles.

In patients with type Ⅲ HL, the apo E2/E2 phenotype may continuously prevent the liver uptake of small CM remnants, resulting in the accumulation of small CM remnants, and lipid-lowering drugs do not improve this uptake.

High fasting apo B-48 and the accumulation of small CM remnants should be treated carefully and reduced using a variety of nutritional and pharmacological approaches. It was reported that atorvastatin markedly improved the postprandial increase in apo B-48-containing lipoproteins in patients with type Ⅲ HL[1]. The apo B-48/TG ratio remained high in patients with type Ⅲ HL in the current study despite appropriate lipid-lowering treatment with fibrates or statins (**Table 1**, **2**, and **3**), which implied that fibrate and statin therapies could not decrease small CM remnants that are clearly atherogenic. For a sufficient decrease in CMs and CM remnants in patients with type Ⅲ HL, the intestinal production of CM must be suppressed along with the increase in LPL activity. In our recent study, we reported that an intestinal cholesterol transporter inhibitor, ezetimibe, improves postprandial HL in patients with type Ⅱb HL[30], possibly due to a reduction in intestinal cholesterol uptake and the formation of apoB-48 and CMs[31]. Combination therapy with fibrates and ezetimibe may more effectively decrease CMs and small CM remnants and may prevent the development of atherosclerotic plaques in patients with type Ⅲ HL.

Limitation of the Study

The present study was performed with a small number of patients with type Ⅲ HL ($n=12$) because it is a very rare familial disorder. It may be necessary to examine whether the apo B-48/TG ratio is useful for detecting type Ⅲ HL with a large-scale study.

Conclusion

In conclusion, these data suggest that the apo B-48/TG ratio is a novel and useful marker for detecting type Ⅲ HL, even after the initiation of lipid-lowering therapy.

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Disclosure

S. Yamashita is a member of Fujirebio's Advisory Boards (Tokyo, Japan). He has received compensation for travel expenses for Conference Lectures from this company. The co-authors do not have anything to disclose.

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Importance of endothelial NF-κB signalling in vascular remodelling and aortic aneurysm formation

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Aims Vascular remodelling and aortic aneurysm formation are induced mainly by inflammatory responses in the adventitia and media. However, relatively little is known about the mechanistic significance of endothelium in the pathogenesis of these vascular disorders. The transcription factor nuclear factor-kappa B (NF-κB) regulates the expressions of numerous genes, including those related to pro-inflammatory responses. Therefore, to investigate the roles of endothelial pro-inflammatory responses, we examined the impact of blocking endothelial NF-κB signalling on intimal hyperplasia and aneurysm formation.

Methods and results To block endothelial NF-κB signalling, we used transgenic mice expressing dominant-negative IκBα selectively in endothelial cells (E-DNIκB mice). E-DNIκB mice were protected from the development of cuff injury-induced neointimal formation, in association with suppressed arterial expressions of cellular adhesion molecules, a macrophage marker, and inflammatory factors. In addition, the blockade of endothelial NF-κB signalling prevented abdominal aortic aneurysm formation in an experimental model, hypercholesterolaemic apolipoprotein E-deficient mice with angiotensin II infusion. In this aneurysm model as well, aortic expressions of an adhesion molecule, a macrophage marker, and inflammatory factors were suppressed with the inhibited expression and activity of matrix metalloproteinases in the aorta.

Conclusion Endothelial NF-κB activation up-regulates adhesion molecule expression, which may trigger macrophage infiltration and inflammation in the adventitia and media. Thus, the endothelium plays important roles in vascular remodelling and aneurysm formation through its intracellular NF-κB signalling.

Keywords NF-κB • Angiotensin II • Inflammation • Atherosclerosis • Abdominal aortic aneurysm

1. Introduction

The endothelium, a single cell layer comprising the vascular wall, forms an interface between vascular structures and blood. There is growing evidence indicating the importance of the endothelium in the maintenance of vessel walls and circulatory function. Endothelial cells produce and react to a wide variety of inflammation-related mediators, including cytokines, growth factors, and adhesion molecules.[1,2] Furthermore, endothelial dysfunctions are involved in the development of many diseases, including atherosclerosis, hypertension, and other inflammatory disorders.[3] Herein, we focused on the role of the endothelium in the pathogenesis of vascular remodelling and aortic aneurysm formation, which are considered to be caused mainly by inflammatory responses in the adventitia and/or media.[4,5]

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Vascular inflammation is a crucial pathological event in various vascular diseases such as angioplastic restenosis and aneurysm formation.[5–7] Intimal hyperplasia is an important feature of angioplastic restenosis and involves excessive accumulation of smooth muscle cells (SMCs) and deposition of extracellular matrix in the intimal layer. The cuff injury model is commonly used to study this type of vascular remodelling. Peri-vascular cuff placement induces adventitial inflammation, leading to medial SMC migration and resultant intimal hyperplasia.[8] Furthermore, chronic inflammation in the media and adventitia also plays a key role in the pathogenesis of aortic aneurysm.[6] A well-established model of abdominal aortic aneurysm (AAA) progression is angiotensin II (AngII) infusion into hypercholesterolaemic apoE$^{-/-}$ (apolipoprotein E-deficient) mice for 4 weeks.[9] In these mice, aneurysmal tissues were characterized by recruitment and infiltration of monocytes/macrophages, proliferation of SMCs, degradation of extra-matrix components including elastin and collagen, and increments in expressions and activities of matrix metalloproteinases (MMPs).[10] However, relatively little is known about the mechanistic importance of endothelial cells in these inflammatory lesions, such as intimal hyperplasia and AAA formation.

Herein, we investigated the roles of endothelial pro-inflammatory responses in these adventitial and medial inflammatory disorders. Nuclear factor-kappa B (NF-κB) is a transcription factor implicated in the processes of both inflammatory responses and oxidative stress.[11] NF-κB is maintained in the cytoplasm in a non-activated form by association with an inhibitor subunit, IκB, when inflammatory stimuli are absent. In response to inflammatory stimuli, proteolysis of IκB exposes a nuclear recognition site of NF-κB and then stimulates it to move into the nucleus, resulting in mRNA expressions of the genes for numerous cytokines, growth factors, and adhesion molecules.[11] We generated transgenic mice overexpressing the dominant-negative form of IκBα under the Tie2 promoter/enhancer (E-DNIκB mice). These mice exhibited functional inhibition of NF-κB signalling specifically in endothelium and prevented obesity- and age-related insulin resistance and enhanced longevity.[12] In addition, inhibition of endothelial NF-κB signalling reportedly attenuates hypertension-induced renal damage,[13] high-fat-diet-induced atherosclerosis,[14] and septic shock-induced vascular dysfunction,[15,16] indicating the pathological importance of endothelial NF-κB signalling. In the present study, using E-DNIκB mice, we examined whether and how the blockade of the NF-κB pathway in endothelial cells affects vascular remodelling and AAA formation. E-DNIκB mice are remarkably protected from the development of cuff injury-induced intimal hyperplasia as well as experimental AAA formation. Thus, the endothelium plays important roles in the promotion of vascular remodelling and aortic aneurysm formation through its intracellular NF-κB pathway.

2. Methods

2.1 Animals

All experiments were performed in conformity with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication, 8th edition, 2011) and the protocols were reviewed and approved by the Institutional Committee for Use and Care of Laboratory Animals of Tohoku University, which was granted by Tohoku University Ethics Review Board (No. 76-21-66). The animals were housed in an air-conditioned environment, with a 12 h light–dark cycle. Transgenic mice (E-DNIκB mice) overexpressing the dominant-negative form of human IκBα (DNIκB), with alanine substitutions of two serine residues (32 and 36), under the Tie2 promoter/enhancer were generated and were backcrossed for at least 10 generations with C57BL/6J mice.[12]

In the experimental procedures, mice were anaesthetized by intraperitoneal injection with medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg). We added these anaesthetic agents if the mice moved to pain at operative time. We, thus, did not detect any movement of mice to pain, and their heart rate and respiratory rate were also stable during the procedures. Mice were sacrificed by cervical dislocation.

For the AAA experiments, E-DNIκB mice were crossed with apoE$^{-/-}$ mice with the C57BL/6J background[17] (The Jackson Laboratory, ME, USA) to generate E-DNIκB;apoE$^{-/-}$ mice. All experiments in this study were performed to allow comparison with littermate control mice.

2.2 Vascular injury by cuff placement around femoral arteries

Peri-vascular cuff placement surgery was carried out in male mice at 8 weeks of age as described previously.[8] After mice were anaesthetized by intraperitoneal injection with medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg), the right femoral artery was dissected from its surroundings, and vascular injury was induced by placing a 2.0 mm polyethylene PE-50 tube (inner diameter 0.58 mm; outer diameter 0.965 mm; BD Bioscience, CA, USA) around the right femoral artery. The contralateral artery served as an uninjured control. Arterial gene expressions were analysed by real-time polymerase chain reaction (RT-PCR) 7 days after cuff placement surgery. Vessels were isolated and processed for histological analysis 21 days after cuff placement surgery. The middle segment of the artery was cut at an interval of 50 μm and the areas covered by the neointima and media were quantified by Scion Image software analysis (Scion, MD, USA) of the digitized microscopic images.

2.3 Analysis and quantification of AAAs

Eight-to-16-week-old male E-DNIκB;apoE$^{-/-}$ ($n = 21$) and littermate apoE$^{-/-}$ control ($n = 20$) mice were subjected to a subcutaneous infusion of AngII (Sigma-Aldrich, MO, USA) (1000 ng/kg/min) for a period of 28 days via Alzet osmotic minipumps (model 1004; DURECT). After mice were anaesthetized by intraperitoneal injection with medetomidine (0.3 mg/kg), midazolam (4 mg/kg), and butorphanol (5 mg/kg), the minipump was implanted subcutaneously in the murine mid-scapular region through an incision in the subdorsal region.[9] The maximum width of the abdominal aorta was quantified by Scion Image software analysis. Aneurysm incidence was determined based on an increase in the external width of the suprarenal aorta of at least 50% compared with that in saline-infused mice.

2.4 Immunohistochemistry and histological analysis

For whole-body perfusion fixation with 100 mmHg pressure, the chest was opened immediately after death, and the heart punctured with a 25 gauge cannula for the infusion of phosphate-buffered saline (PBS) under physiological pressure. The blood was drained through an incision of the inferior vena cava. After 5 min, PBS was replaced with 10% buffered formaldehyde and the mice perfused for an additional 5 min prior to dissection and overnight post-fixation with 10% buffered formaldehyde. Tissues were dehydrated through various concentrations of ethanol and embedded in paraffin as described previously.[18] Excised whole and thoracic aortae and femoral arterial tissues were subjected to staining with elastica and haematoxylin–eosin and immunostaining with antibodies against Mac-3 (BD Bioscience) or the p65 subunit of NF-κB (Santa Cruz Biotechnology, CA, USA) as described previously.[18] The areas of the intima and media were measured and averaged in five cross-sections per mouse.

2.5 Blood analysis

Blood glucose, plasma insulin, total cholesterol, and triglyceride concentrations were determined as described previously.[19] Plasma thiobarbituric acid-reactive substance (TBARS) levels were measured with a TBARS Assay Kit (Cayman Chemical Co., MN, USA).

2.6 Blood pressure measurement

Systolic blood pressure in the conscious state was measured by the indirect tail cuff method, using a model MK-2000 BP monitor for mice and rats (Muromachi Kikai, Tokyo, Japan) as described previously.[20] At least six readings were obtained for each experiment, and a mean value was assigned to male E-DNIκB;apoE$^{-/-}$ ($n = 13$) and littermate control apoE$^{-/-}$ ($n = 11$) mice.

2.7 Gelatin zymography

Aortic proteins were extracted using a gelatin-zymography kit (Primary Cell, Sapporo, Japan) as previously described.[10] The molecular sizes of gelatinolytic activities were determined according to the manufacturer's instructions. The sum of MMP-2 and Pro-MMP-2 was measured as total MMP-2 activity.

2.8 Quantitative RT-PCR-based gene expressions

Whole aortae and femoral arteries were perfused and rinsed with physiological saline. Total RNA was extracted using an RNAeasy micro kit (Qiagen, CA, USA). Quantitative RT-PCR was performed as previously described.[18] cDNA synthesis was performed with a Cloned AMV First Strand Synthesis Kit (Invitrogen, CA, USA), using 1.0 μg of total RNA. cDNA synthesized from total RNA was evaluated using a quantitative RT-PCR system (Light Cycler Quick System 350S; Roche Diagnostics GmbH). The relative amounts of mRNA were calculated with β-actin mRNA as the invariant control. The oligonucleotide primers are described in Supplementary material online, *Table S1*.

2.9 Statistical analysis

Data are expressed as means ± SEM. All statistical analyses were performed with the Ekuseru-Tokei 2010 statistical software (Social Survey Research Information Co., Ltd, Tokyo, Japan). All data were tested for normality by the Kolmogorov–Smirnov test. When data were normally distributed, the statistical significance of differences was assessed with one-way ANOVA. The Mann–Whitney *U* test was applied when data were not normally distributed. *P*-values of <0.05 were considered to be statistically significant.

3. Results

3.1 Cuff injury-induced intimal hyperplasia was suppressed in E-DNIκB mice

To elucidate the roles of endothelial NF-κB signalling in vascular remodelling, we examined cuff injury-induced intimal hyperplasia in E-DNIκB mice, i.e. transgenic mice overexpressing the dominant-negative form of IκBα under the Tie2 promoter/enhancer.[12] As reported previously,[12] immunoblotting revealed no detectable expression of the dominant-negative form of IκBα in macrophages, whereas it was clearly detected in the aorta and lung in which the endothelium is abundant (see Supplementary material online, *Figure S1*). Endogenous IκBα proteins in the lung were degraded after TNF-α stimulation in a dose-dependent manner, whereas DNIκBα remained essentially intact without degradation after TNF-α stimulation (see Supplementary material online, *Figure S2A*). In addition, TNF-α-stimulated up-regulation of VCAM-1 in endothelial cells was markedly suppressed in E-DNIκB mice compared with the wild-type controls (see Supplementary material online, *Figure S2B*). In contrast, TNF-α stimulation similarly up-regulated interleukin (IL)-6, a target gene of NF-κB, in peritoneal macrophages isolated from the wild-type and E-DNIκB mice (see Supplementary material online, *Figure S2C*). These findings demonstrate the functional blockade of NF-κB signalling selectively in endothelial cells of the E-DNIκB mice used in this study.

E-DNIκB mice showed no obvious differences in metabolism from control mice under normal chow-fed conditions. At 8 weeks of age, body weights, fasting blood glucose, and insulin levels were similar in E-DNIκB mice and their wild-type littermates. Neither total cholesterol nor triglyceride levels differed significantly between the two. In addition, blood pressures were similar in these two groups of mice (see Supplementary material online, *Table S2*). Under these conditions, a peri-vascular cuff was placed around the femoral artery, followed by examination of morphometric changes in the artery after 21 days.

No apparent histological differences were observed in uninjured femoral arteries between E-DNIκB and wild-type littermate mice (*Figure 1A*). In contrast, in cuff-injured femoral arteries, E-DNIκB mice exhibited apparent suppression of intimal hyperplasia (*Figure 1A*). Intimal hyperplasia, when quantified as the intimal area and intima/media ratio, was also significantly decreased (by 44.5 and 47.5%, respectively) in E-DNIκB mice compared with littermate controls (*Figure 1B* and *C*). Thus, the blockade of endothelial NF-κB signalling suppressed vascular remodelling.

3.2 Endothelial NF-κB blockade suppressed gene expressions of inflammation- and SMC proliferation-related factors

Intimal hyperplasia in the arterial wall is reportedly attributable to increased inflammatory reactions induced by interactions between recruited leucocytes and migrating SMCs.[21] Therefore, we next examined local expressions of genes related to vascular remodelling in cuff-injured femoral arteries 7 days after cuff placement. In cuff-injured femoral arteries, expressions of inflammatory factors, such as monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-1β, and IL-6, were markedly suppressed in E-DNIκB mice compared with controls (*Figure 2A*), suggesting suppressed arterial inflammation. Compatible with this notion, arterial expression of F4/80, a macrophage marker, was significantly decreased in E-DNIκB (*Figure 2B*). In addition, endothelial DNIκB expression inhibited expressions of vascular SMC proliferation-related factors, such as platelet-derived growth factor-B and stromal cell-derived factor-1α (also known as CXCL12) (*Figure 2C*). These findings suggest cuff injury-induced inflammation and SMC proliferation to be suppressed in E-DNIκB mice. Furthermore, arterial expressions of cellular adhesion molecules, such as VCAM-1 and ICAM-1, were remarkably decreased in E-DNIκB mice (*Figure 2D*). Anti-oxidant enzymes, such as manganese superoxide dismutase (MnSOD) and glutathione-*S*-transferase (GST), which are up-regulated in response to oxidative stress, were attenuated by endothelial DNIκB expression (*Figure 2E*). These findings suggest that the blockade of NF-κB signalling in the endothelium inhibits up-regulation of vascular adhesion molecules and the resultant

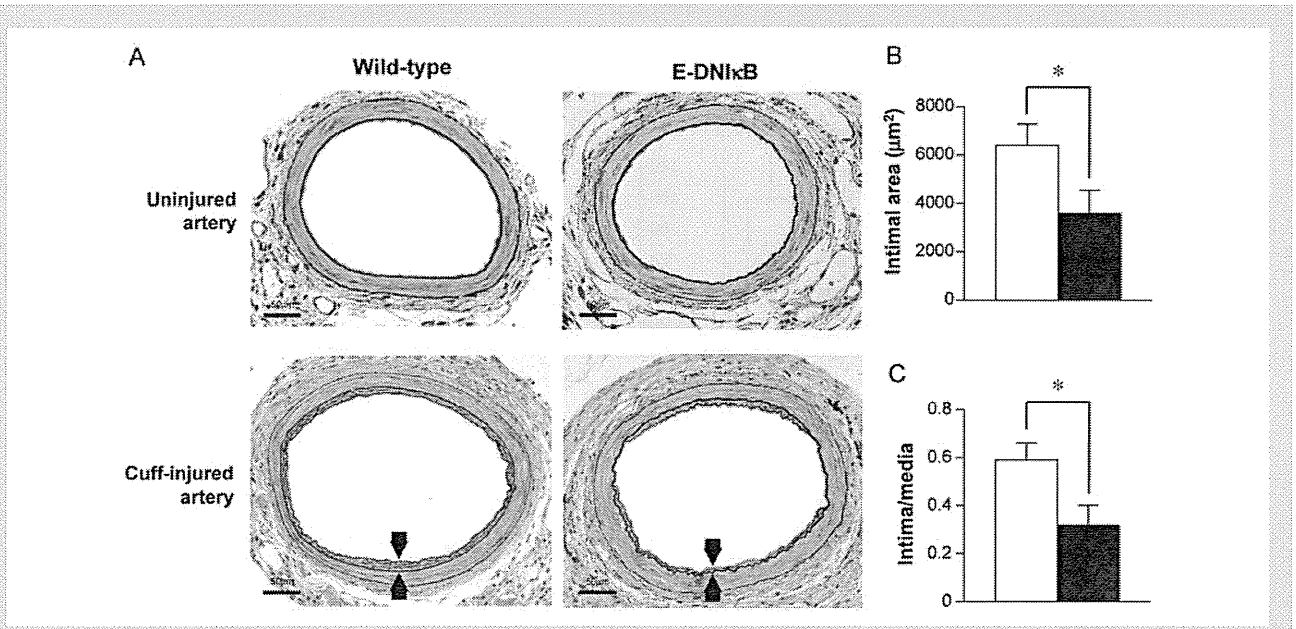

Figure 1 Cuff injury-induced intimal hyperplasia was inhibited in E-DNIκB mice. Peri-vascular cuff placement surgery was carried out on male E-DNIκB and littermate wild-type mice at 8 weeks of age, followed by histological analysis after 21 days. (*A*) Representative histological findings of uninjured (upper) and cuff-injured (lower) femoral arteries from wild-type (left) and E-DNIκB (right) mice. Magnification: ×200. Scale bars indicate 50 μm. Arrows indicate the intimal hyperplasia induced by cuff injury. (*B*) Intimal area of cuff-injured femoral arteries of wild-type (white bar, $n = 6$) and E-DNIκB (black bar, $n = 4$) mice. (*C*) Intimal-to-medial area ratios of cuff-injured femoral arteries of wild-type (white bar, $n = 6$) and E-DNIκB (black bar, $n = 4$) mice. Data are presented as means ± SEM. $^*P < 0.05$, assessed by one-way ANOVA.

macrophage infiltration, which is the mechanism underlying suppression of cuff injury-induced adventitial inflammation.

3.3 E-DNIκB mice were protected from experimental AAA formation

In the cuff injury model, adventitial inflammation is considered to be a major cause of medial SMC migration to the subendothelial space.[22] We next examined the contribution of NF-κB signalling in the endothelium to aneurysm formation, another aortic medial and adventitial lesion. E-DNIκB mice were crossed with apoE$^{-/-}$ mice, resulting in the generation of E-DNIκB;apoE$^{-/-}$ mice. E-DNIκB;apoE$^{-/-}$ mice and control apoE$^{-/-}$ mice were started on a 28-day AngII infusion (1000 ng/kg/min), followed by abdominal aorta analyses.

Body weight, blood glucose and serum levels of insulin, triglycerides, and total cholesterol were similar in E-DNIκB;apoE$^{-/-}$ mice and control apoE$^{-/-}$ mice after the 28-day AngII infusion (see Supplementary material online, *Table S3*). Although systolic blood pressure was significantly elevated in control apoE$^{-/-}$ mice, blood pressure elevation was blocked by endothelial DNIκB expression (*Figure 3A*). Notably, AAA formation after AngII infusion was attenuated in E-DNIκB;apoE$^{-/-}$ mice compared with control apoE$^{-/-}$ mice (*Figure 3B*). AngII infusion for 28 days led to AAA formation at an incidence of 85.0% (17 out of 20) in control apoE$^{-/-}$ mice, whereas AAA was detected in only 19.0% (4 out of 21) of E-DNIκB;apoE$^{-/-}$ mice. The maximal abdominal aortic diameter of E-DNIκB;apoE$^{-/-}$ mice (2.06 ± 0.22 mm) was significantly decreased compared with that of control apoE$^{-/-}$ mice (1.49 ± 0.08 mm) (*Figure 3C*). Thus, the blockade of endothelial NF-κB signalling clearly prevented AAA progression in this experimental model of AngII infusion into hypercholesterolaemic mice.

3.4 AngII-induced macrophage infiltration, aortic inflammation, and oxidative stress were markedly reduced by blockade of NF-κB signalling in endothelial cells

To further delineate the effects of the endothelial NF-κB blockade on AAA formation, we next performed morphological and immunohistochemical studies of vessel-wall constituents. Cross-sectional histology with elastica and haematoxylin–eosin staining revealed that AngII-induced lumen dilatation and medial elastin breaks, which were observed in the abdominal aortae in apoE$^{-/-}$ controls, were remarkably attenuated in the aortae in E-DNIκB;apoE$^{-/-}$ mice (*Figure 4A*). Furthermore, immunohistochemistry with anti-Mac-3 also revealed massive infiltration of macrophages in the media and adventitia of the aortic walls of apoE$^{-/-}$ controls, whereas macrophage infiltration was markedly inhibited in E-DNIκB;apoE$^{-/-}$ mice (*Figure 4B*).

These findings were compatible with aortic gene expressions. Aortic F4/80 expression was significantly decreased in E-DNIκB;apoE$^{-/-}$ mice (*Figure 5A*). In addition, aortic expressions of inflammatory factors, such as MCP-1 and IL-1β (*Figure 5B*), and the cellular adhesion molecule VCAM-1 (*Figure 5C*) were significantly decreased in E-DNIκB;apoE$^{-/-}$ mice. Furthermore, an anti-oxidant enzyme MnSOD tended to be decreased and GST was markedly suppressed (*Figure 5D*), suggesting decreased oxidative stress. Supporting this notion, plasma levels of TBARS, a marker of oxidative stress, were significantly decreased in E-DNIκB;apoE$^{-/-}$ mice (*Figure 5E*). Thus, oxidative stress in the aorta

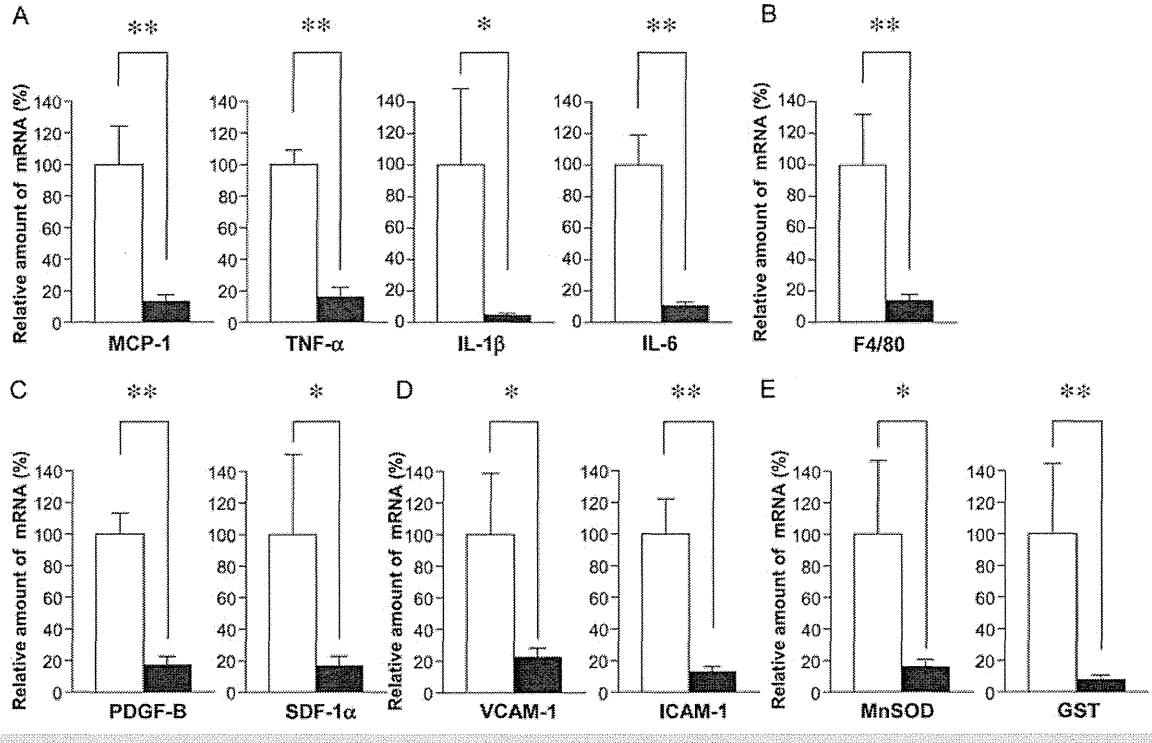

Figure 2 Expressions of inflammation- and SMC proliferation-related factors and adhesion molecules in cuff-injured arteries were suppressed in E-DNIκB mice. Arterial expressions of pro-inflammatory cytokines (*A*), a macrophage marker (*B*), vascular SMC proliferation-related factors (*C*), cellular adhesion molecules (*D*), and anti-oxidant enzymes (*E*) were quantified by RT-PCR 7 days after cuff placement in wild-type (white bars, $n = 6$) and E-DNIκB (black bars, $n = 6$) mice. Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, assessed by one-way ANOVA.

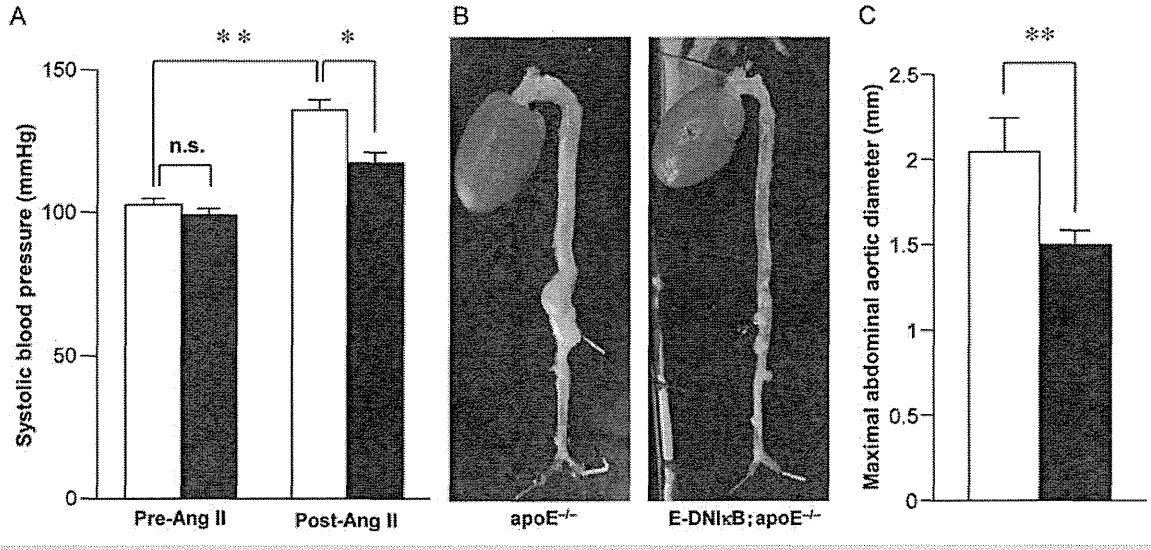

Figure 3 AAA formation was inhibited by the blockade of endothelial NF-κB signalling. E-DNIκB;apoE$^{-/-}$ (black bars, $n = 21$) and littermate apoE$^{-/-}$ control (white bars, $n = 20$) mice were subjected to a subcutaneous infusion of AngII for 28 days. (*A*) Systolic blood pressures of control apoE$^{-/-}$ (white bars, $n = 11$) and E-DNIκB;apoE$^{-/-}$ (black bars, $n = 13$) mice before and after 4-week AngII infusion. (*B*) Representative macroscopic findings of the abdominal aortae from control apoE$^{-/-}$ (left) and E-DNIκB;apoE$^{-/-}$ (right) mice. (*C*) Maximal abdominal aortic diameters from control apoE$^{-/-}$ mice and E-DNIB;apoE$^{-/-}$ mice. Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, assessed by one-way ANOVA.

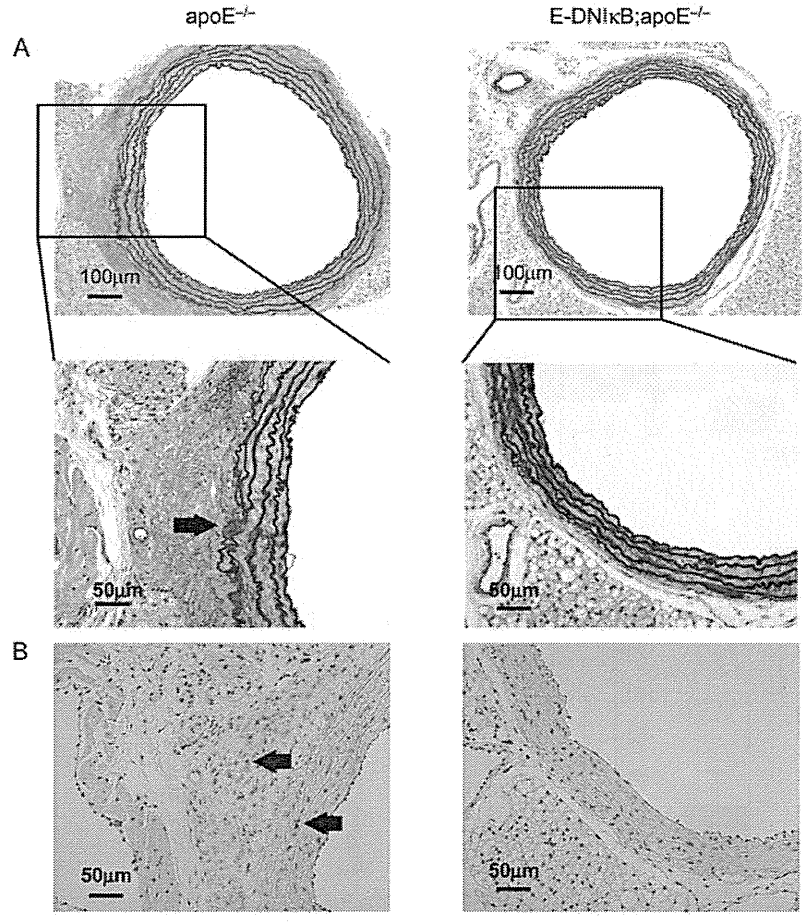

Figure 4 Medial elastin breaks and macrophage infiltration were markedly inhibited by the blockade of endothelial NF-κB signalling. E-DNIκB;apoE$^{-/-}$ and littermate apoE$^{-/-}$ control mice were subjected to a subcutaneous infusion of AngII for 28 days. (*A*) Aortic sections were counterstained with elastica and haematoxylin–eosin. The arrow indicates breaks in the elastic fibres of apoE$^{-/-}$ control mice. Magnification: (*A* upper): ×100; (*A* lower): ×200. (*B*) Aortic sections were immunostained with anti-mac3 antibody. Magnification: ×200. The arrows indicate Mac 3-positive cells. Results are representative of data obtained from six mice per group.

as well as the whole body was decreased in E-DNIκB;apoE$^{-/-}$ mice. Therefore, the blockade of NF-κB signalling in endothelial cells alone has a strong inhibitory impact on macrophage infiltration, aortic inflammation, and oxidative stress, thereby preventing aortic aneurysm formation.

3.5 Activation and expression of MMPs were inhibited in the aortae of E-DNIκB;apoE$^{-/-}$ mice

MMPs, which are zinc-dependent endopeptidases, have been shown to be responsible for the destruction of the orderly elastin and collagen network of the aorta.[23] In fact, activated MMP-2 and MMP-9 levels are reportedly elevated in human AAA tissue.[10] Macrophage-derived MMP-9 and SMC-derived MMP-2 are reportedly both required and work in concert, resulting in aneurysm progression.[10] Therefore, we next analysed the activities of aortic MMP-2 and MMP-9 by the gelatin zymography technique. The AngII-induced activities of aortic MMP-2 and pro-MMP-2, as well as those of pro-MMP-9, were markedly suppressed in E-DNIκB;apoE$^{-/-}$ mice compared with control apoE$^{-/-}$ mice (*Figure 6A*). RT-PCR revealed that expressions of MMP-2 and MMP-9 were decreased (*Figure 6B*). Thus, decreased MMP-2 and MMP-9 expressions/activities are involved in the reduced incidence and severity of experimental aneurysm formation observed in E-DNIκB;apoE$^{-/-}$ mice.

4. Discussion

To investigate the roles of endothelial pro-inflammatory responses in adventitial and medial inflammatory disorders, we used E-DNIκB mice in the present study. We previously reported that, in E-DNIκB mice, DNIκBα was selectively expressed in endothelial cells and resistant to degradation, thereby inhibiting the movement of NF-κB to the nucleus even after TNF-α stimulation.[12] Furthermore, pro-inflammatory responses were suppressed in endothelial cells, but not in circulating cells, obtained from E-DNIκB mice.[12] Although only a small fraction of the circulating leucocytes and marrow cells were reported

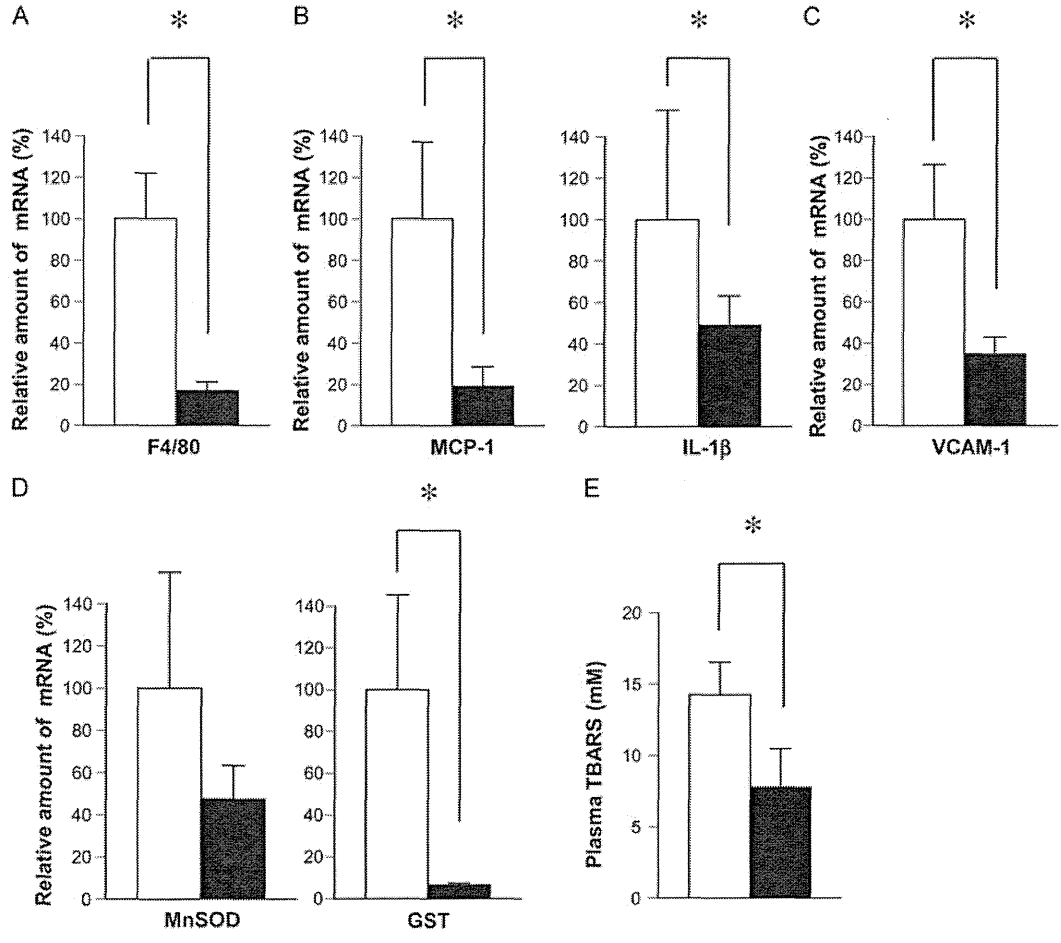

Figure 5 Suppression of inflammation and oxidative stress by the blockade of endothelial NF-κB signalling. (*A–D*) Aortic expressions of a macrophage marker (*A*), pro-inflammatory cytokines (*B*), a cellular adhesion molecule (*C*), and anti-oxidant enzymes (*D*) were quantified by RT-PCR after 28-day AngII infusions into apoE$^{-/-}$ control (white bars) and E-DNIκB;apoE$^{-/-}$ (black bars, $n = 5–6$ per group) mice. (*E*) Plasma TBARS concentrations were measured in control apoE$^{-/-}$ and E-DNIκB;apoE$^{-/-}$ mice. Data are presented as means ± SEM. *$P < 0.05$, assessed by one-way ANOVA.

to weakly express the Tie2/Tek gene,[24] Tie2-expressing monocytes, including macrophages, are preferentially extravasate in tumours and regenerating tissues. In our models, DNIκBα was not detected by immunoblotting in isolated peritoneal macrophages. In addition, macrophages from E-DNIκB mice did not suppress TNF-α-stimulated expression of IL-6, a target gene of NF-κB. Although we cannot completely rule out that a very small amount of DNIκB was expressed in macrophages or other circulating cells, these data suggest that NF-κB signalling is functionally blocked selectively in endothelial cells of the E-DNIκB mice used in this study and that effects of DNIκB in non-endothelial cells, if even present, were very small.

The first important result obtained in this study is that the endothelial blockade of intracellular NF-κB signalling markedly suppressed intimal hyperplasia. Peri-vascular cuff placement stimulates arteries from outside of the adventitia and the resultant adventitial inflammatory cell infiltration is considered to cause migration and accumulation of medial SMCs.[4] In the present study, interestingly, the blockade of NF-κB signalling only in the endothelium suppressed these adventitial and medial events. In the peri-vascular cuff-injured arteries of E-DNIκB mice, expressions of endothelial adhesion molecules, such as VCAM-1 and ICAM-1, were markedly decreased, in association with reduced macrophage marker expression. Thus, the endothelium-macrophage interaction, triggered by endothelial adhesion molecules, was markedly inhibited in E-DNIκB mice, which is likely involved in the mechanism underlying suppression of adventitial inflammation and the resultant vascular remodelling. Therefore, up-regulation of cellular adhesion molecules in the endothelium is likely to be the early important event in cuff injury-induced vascular remodelling, and this step appears to be regulated by NF-κB signalling in endothelial cells. Thus, the endothelium, which lines the entire vascular system in a single cell layer, may play important roles in inflammatory responses, thereby inducing vascular remodelling. In addition to the arterial lumen, the adventitial vasa vasorum, which is enriched in the endothelium, acts as a conduit for the entry of inflammatory mediators and circulating cells into the vessel wall. Growing evidence supports the 'outside–in' theory, in which vascular inflammation is initiated in the adventitia and progresses inward towards the intima.[25] The blockade of NF-κB signalling in the adventitial vasa

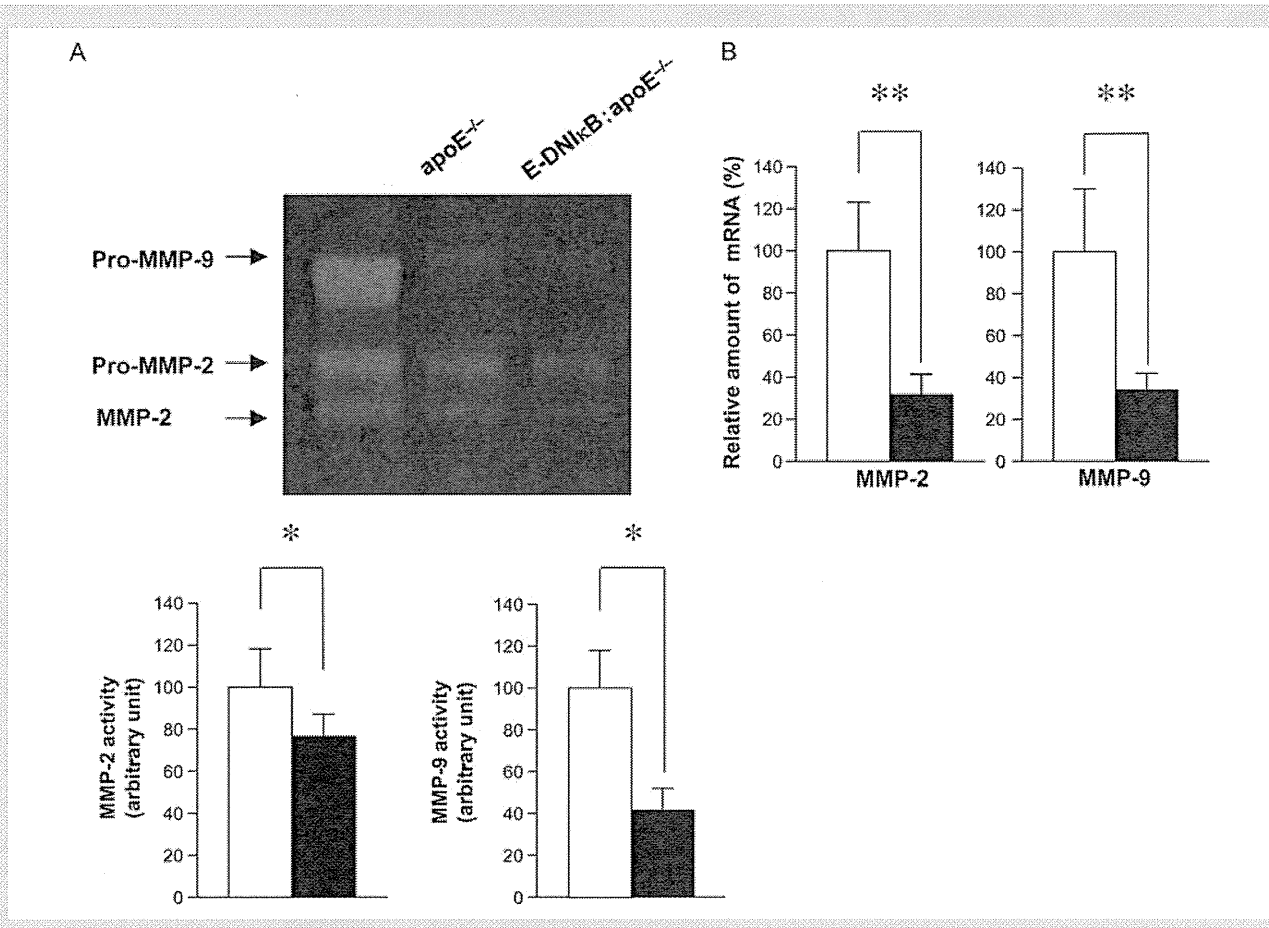

Figure 6 Suppression of MMP activation by the blockade of endothelial NF-κB signalling. (*A*) Representative zymographic analyses for MMP activities in aortae from control apoE$^{-/-}$ and E-DNIκB;apoE$^{-/-}$ mice. Markers for MMP-2, pro-MMP-2 and pro-MMP-9 are shown in the left lane. Gelatin zymographic activities of MMP-2 and MMP-9 in aortae from control apoE$^{-/-}$ and E-DNIκB;apoE$^{-/-}$ mice were quantified by densitometric analysis. (*B*) Aortic expressions of MMP-2 and MMP-9 were quantified by RT-PCR after 28-day AngII infusions into apoE$^{-/-}$ control (white bars, $n = 6$) and E-DNIκB;apoE$^{-/-}$ (black bars, $n = 8$) mice. Relative amounts of mRNA in aortae from control apoE$^{-/-}$ and E-DNIκB;apoE$^{-/-}$ mice. Data are presented as means ± SEM. *$P < 0.05$, **$P < 0.01$, assessed by one-way ANOVA.

vasorum endothelium might inhibit the entry of macrophages and inflammatory mediators, thereby contributing to the vascular remodelling suppression observed in E-DNIκB mice.

Another important finding of the present study is that NF-κB signalling in the endothelium is the key step in aortic aneurysm formation. Aneurysm formation is mainly influenced by the physical and biochemical vascular inflammatory responses in the media and adventitia through several mechanisms.[6] Infiltrating macrophages/leucocytes are major sources of proteinases, such as MMPs, that degrade structural proteins, including elastin, collagen, and laminin, thereby weakening the aortic wall.[6,10] In addition, infiltrating immune cells may exacerbate tissue injury through the release of inflammatory cytokines, leading to further recruitment of immune cells and induction of SMC apoptosis.[26] Apoptotic SMCs play major roles in aortic extracellular matrix production as well as additional protease release, contributing to matrix degradation.[10,26] In addition, oxidative stress is reportedly involved in aneurysm progression.[27] In the present study, the blockade of endothelial NF-κB signalling suppressed aortic expressions of inflammatory factors and oxidative stress markers in an experimental AAA model. In addition, VCAM-1 and F4/80 expressions were markedly decreased in the aortae of E-DNIκB;apoE$^{-/-}$ mice, indicating the suppression of the endothelium–leucocyte interaction. Thus, endothelial NF-κB activation may induce the endothelium–leucocyte interaction, triggering whole-aorta inflammation. Furthermore, aortic expressions and activations of MMP-2 and MMP-9 were also inhibited by the endothelial blockade of NF-κB signalling. These findings, taken together, suggest that the endothelial pro-inflammatory response is a major early event in aortic aneurysm formation. This notion that the endothelium–leucocyte interaction triggers AAA formation is supported by previous reports indicating that global P-selectin deficiency and haematopoietic CCR2 deficiency attenuate experimental aortic aneurysm formation.[28,29]

AngII reportedly induces intracellular NF-κB activation.[30] However, although a growing body of evidence has accumulated regarding AngII signalling in SMCs, much less is known about endothelial AngII signal transduction and function.[31] The present study shows the specific importance of the endothelium, which eventually modulates inflammation in the adventitia and media, through intracellular NF-κB

signalling. Furthermore, AngII-induced elevation of blood pressure was almost completely absent in E-DNIκB;apoE$^{-/-}$ mice, suggesting that endothelial NF-κB activation mediates AngII-related hypertension. Thus, NF-κB signalling in the endothelium plays mechanistic roles in various aspects of the development of vascular disorders. Since cohort and epidemiological studies have revealed the relevance of hypertension to AAA,[32] this preventive effect on blood pressure elevation might participate in the suppression of AAA formation observed in E-DNIκB;apoE$^{-/-}$ mice. However, Cassis *et al.* [33] recently reported based on the three data sets described below that blood pressure elevation *per se* is not a major determinant of AAA formation in this model. Blood pressure elevation in apoE$^{-/-}$ mice infused with norepinephrine did not induce AAA formation. Subpressor infusion of AngII induced AAA development in apoE$^{-/-}$ mice. Furthermore, lowering systolic blood pressure by the administration of hydralazine to AngII-infused apoE$^{-/-}$ mice did not prevent AAA formation. Thus, the suppression of inflammatory responses, rather than the prevention of hypertension, is likely to be the main contributor to preventive effects of the endothelial NF-κB blockade on AAA formation.

In conclusion, this study provides strong evidence of the major impact of the endothelium on vascular remodelling and AAA formation through intracellular NF-κB signalling. Endothelial NF-κB signalling up-regulates adhesion molecules, triggering macrophage infiltration and the resultant vascular inflammation in the adventitia and media. Therefore, NF-κB activation is a major early event in the pathogenesis of these inflammation-related vascular diseases, making the blockade of endothelial NF-κB signalling a promising strategy for preventing vascular inflammation and dysfunction, especially aneurysm formation as well as angioplastic restenosis.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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