A recent study evaluated 238 advanced heart failure patients referred for cardiac transplantation evaluation who had a cTnI assay drawn at the time of initial presentation. Patients with acute myocardial infarction were excluded. Detectable cTnI was associated with progressive decline in ejection fraction over time and with increased mortality. Patients with ischaemic and non-ischaemic causes of heart failure were similar in terms of cTnI concentrations and other laboratory values, NYHA class, left ventricular ejection fraction, and haemodynamics.[40]

H-FABP

Recently, Setsuta and colleagues, who previously reported the elevation of cTnT in patients with heart failure,[30] reported that H-FABP was associated with subsequent cardiac events in patients with chronic heart failure caused by DCM, old myocardial infarction, hypertensive heart disease, valvar heart disease, or congenital heart disease.[41] While both cTnT and H-FABP were associated with subsequent cardiac deaths or rehospitalisation for the management of worsening heart failure, H-FABP was much more detectable among patients in NYHA functional class II. H-FABP is a small protein abundant in the cytosol which is readily released into the circulation following myocardial damage. In contrast, most troponins are components of the myofibrillar contractile apparatus, present in small amounts in the cytosol. This may explain the different patterns of increase of these two markers following myocyte injury.

MLC-1

Studies of MLC-1, a 27 kD protein, as a biochemical marker of myocyte injury in patients with heart failure are few. Hansen and colleagues reported that circulating MLC-1 was elevated in some patients in NYHA functional class III and IV, and this increase was associated with a poor prognosis in a clinical trial of flosequinin.[42] Studies are needed to further characterise this marker in patients with heart failure and to distinguish it from the other biochemical markers of myocyte injury described earlier.

USE OF BIOCHEMICAL MARKERS TO MEASURE THE DEGREE OF HEART FAILURE

Since heart failure is a complex clinical syndrome, a single biomarker may not reflect all of its characteristics. The serial and combined measurements of biochemical markers of myocyte injury may open new perspectives in heart failure. Brain natriuretic peptide (BNP) is an amino acid peptide chiefly secreted by the ventricular myocardium in response to strain. The plasma measurement of BNP is being used increasingly in the diagnosis, prognosis, and monitoring of patients with congestive heart failure.[43–45] BNP may be viewed as a marker of myocardial load and cTnT as a marker of myocyte injury. Combining these biochemical markers may provide new insight in the management of heart failure. In our small study of patients presenting with decompensated heart failure, approximately one third had initial concentrations of cTnT within normal limits. While BNP decreased significantly after treatment in all patients, cTnT remained elevated in most patients whose initial concentrations were increased, despite radiographic resolution of pulmonary congestion.[29] We hypothesise that a first therapeutic goal should consist of relief of circulatory congestion and lowering of BNP, and a second goal be the mitigation of myocyte injury and lowering of cTnT. Recently, Ishii and colleagues reported that cTnT concentrations > 0.033 ng/ml and BNP concentrations > 440 pg/ml, at the time of admission to the coronary care unit, correlated with significantly higher rates of cardiac events among 98 consecutive patients hospitalised for management of worsening chronic heart failure.[35]

At this time, the relative contributions of cTnT, cTnI, H-FABP, and MLC-1 in patients with heart failure remain unclear. The different half-lives, molecular sizes, and intracellular distributions of these markers may provide detailed information regarding the process of myocyte injury by monitoring the markers in combination. Combinations of markers of myocyte injury and markers of interstitial matrix collagen turnover may also add new information on the process of cardiac remodelling in patients with chronic heart failure.[24]

FUTURE APPLICATIONS OF BIOCHEMICAL MARKERS IN HEART FAILURE

Mechanisms of myocyte injury and biochemical markers

Although these biochemical markers indicate the presence of ongoing myocyte injury in patients with heart failure, the mechanisms of that injury remain unclear. In our study of DCM, the presence of active myocarditis was excluded by endomyocardial biopsies using the Dallas criteria.[25] Furthermore, transverse sections of postmortem cardiac specimens from three patients with DCM with persistently elevated cTnT showed no significant mononuclear cellular infiltration (unpublished data). The mechanism of myocyte injury without cellular infiltration needs to be studied. Adrenergic stimulation, calcium handling abnormalities, the renin–angiotensin system, endothelin, inflammatory cytokines, nitric oxide, oxidative stress, and mechanical stress have been explored as potential contributors to myocyte injury in the setting of heart failure.[46 47] The existence of correlations among these factors with biochemical markers of myocyte injury should be examined in clinical studies to provide important information applicable to the management of heart failure.

Biochemical markers as surrogate end points in heart failure

Since heart failure is a life threatening condition, survival was chosen as the primary end point in the clinical trials which proved the effectiveness of angiotensin–converting enzyme inhibitors, aldosterone antagonists, and β adrenergic blockers.[48] However, large study populations and long study periods are usually required to show a significant effect of treatment on survival. Therefore, the interest in surrogate end points has recently increased, since their use may allow the successful completion of controlled clinical trials with smaller patient populations, within shorter observation periods. Combinations of certain biochemical markers described earlier may represent surrogate endpoints suitable for the design of such trials.[49]

CONCLUSIONS

No guidelines have been issued regarding the monitoring of biochemical markers of myocyte injury as part of the management of chronic heart failure. Recent technological advances will allow the rapid application of these assays in the near future. The real time detection of myocyte injury will render the management of heart failure more precise and effective. It is our expectation that these assays will become the new standards in the monitoring of patients with heart failure.

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Combined Measurements of Cardiac Troponin T and N-Terminal Pro-Brain Natriuretic Peptide in Patients With Heart Failure

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Background To examine the prognostic contribution of combined cardiac troponin T (cTnT) and N-terminal pro-brain natriuretic peptide (NT-proBNP) in patients with heart failure (CHF) in the absence of acute coronary syndrome.

Methods and Results Between July 2001 and March 2002, 71 consecutive patients (mean age=68.4±1.4 years, 37 men), hospitalised for heart failure, were studied during hospitalisation and follow up until December 2002. Serum cTnT and NT-proBNP were measured on admission. Actuarial rates of adverse cardiac events, including sudden or CHF death, or rehospitalisation for CHF during follow up were compared with patients grouped according to initial serum cTnT and/or NT-proBNP concentrations. The adverse cardiac event-free rate among the 20 patients with cTnT≥0.01 ng/ml was significantly lower than the 51 patients with cTnT<0.01 ng/ml ($P<0.05$). Similarly, the adverse cardiac event-free rate among the 36 patients with NT-proBNP≥1,357 pg/ml (median) was significantly lower than the 35 patients with NT-proBNP<1,357 pg/ml ($P<0.01$). The 16 patients with high concentrations of both cTnT and NT-proBNP had a lower adverse cardiac event-free rate than the 31 patients with low cTnT and low NT-proBNP upon commencement of the study ($P<0.005$).

Conclusion Measurements of serum cTnT and NT-proBNP were reliable prognostic markers of adverse cardiac event in patients with CHF. (*Circ J* 2004; **68:** 1160–1164)

Key Words: Heart failure; Pro-brain natriuretic peptide; Prognosis; Troponin

Chronic heart failure (CHF) is associated with a dismal long-term prognosis and remains a major health concern world wide[1,2] While various management strategies have become available, clinical tools to stage CHF remain few. The New York Heart Association (NYHA) functional classification, along with several tests, including chest roentgenogram, echocardiogram, myocardial scintigraphy, cardiopulmonary exercise, and hemodynamic measurements are useful to estimate the degree of CHF, although they are subject to inter-observer variations in interpretation[3,4] Serial measurements of reliable and objective biochemical markers would be advantageous to monitor the long-term prognosis of patients with CHF.

The troponin complex consists of 3 proteins attached to the actin thin filament, known as subunits I, T, and C, which regulate the force and velocity of muscle contraction. Cardiac troponin T (cTnT) is a highly sensitive and specific marker of myocardial injury in acute coronary syndromes, and a revised definition of acute myocardial infarction has been developed, based on rises in cardiac troponins in the blood[5,6] We found that patients with idiopathic dilated cardiomyopathy, who had a particularly poor prognosis, had increased serum concentrations of cTnT in the absence of significant coronary stenoses[7–10] Most patients with poor outcomes had persistently high cTnT. This often occurred during periods when CHF was stabilised by conventional treatment, and there was no evidence of dyspnea, roentgenographic and auscultatory signs of pulmonary congestion[8,9] Therefore, an increase in serum cTnT concentrations seems to be a reliable indicator of ongoing subclinical myocyte injury rather than an indicator

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Table 1 Demographic and Baseline Clinical Characteristics of Study Population (n=71)

Age, mean±SE (years)	*68.4±1.4*
M/F	*37/34*
NYHA functional class I/II/III/IV	*10/22/22/17*
Underlying heart disease	
Dilated cardiomyopathy	*20 (28)*
Hypertrophic cardiomyopathy	*8 (11)*
Ischemic	*8 (11)*
Congenital or valvular	*22 (31)*
Hypertensive	*9 (13)*
Other	*4 (6)*
Oral drug regimen	
β-adrenergic blockade	*24 (34)*
ACEI or ARB	*33 (46)*
Spironolactone	*33 (46)*
Furosemide	*49 (69)*

Unless indicated otherwise, values are number (%) of patients. Other heart diseases include incessant tachyarrythmias (n=2), cardiac amyloidosis (n=1) and restrictive cardiomyopathy (n=1).

ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker; NYHA, New York Heart Association.

Table 2 Mean NT-ProBNP, CK, Age, and LVEF Among Patients With High and Low cTnT Values at Time of Hospital Admission

	NT-proBNP (pg/ml)	CK (IU/L)	Age (years)	LVEF (%)
cTnT high (n=20)	13,260±5,035*	90.2±9.2	68.5±3.5	49.6±3.1
cTnT low (n=51)	1,847±311	91.8±6.1	68.3±1.5	53.9±2.7

*P<0.001, other between-group differences are not statistically significant.
CK, creatine kinase; cTnT, cardiac troponin T; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-brain natriuretic peptide.

Table 3 Comparison Between Patients With and Without Cardiac Decompensation

	Decompensation (+) (n=45)	Decompensation (–) (n=26)
Age, mean±SE (years)	70.4±1.7	64.8±2.4
M/F	21/24	16/10
LVEF (%)	49.5±2.5	58.1±3.6
NYHA functional class I/II/III/IV	0/12/16/17	10/10/6/0
TnT positive (%)	16/45 (35)	4/26 (15)
Mean TnT of positive patients (ng/ml)	0.037±0.004	0.038±0.002
NT-proBNP (pg/ml)	7,233±2,369	1,303±291*
Creatinine (mg/dl)	1.1±0.1	1.0±0.1
Underlying heart disease		
Dilated cardiomyopathy	14 (31)	6 (23)
Ischemic	5 (11)	3 (11)
Congenital or valvular	14 (31)	8 (31)
Hypertensive	8 (17)	1 (4)
Oral drug regimen		
β-adrenergic blockade	12 (27)	12 (46)
ACEI or ARB	22 (48)	11 (42)
Spironolactone	26 (58)	7 (27)*
Furosemide	37 (82)	12 (46)**
Cardiac event (%)	10 (22)	0 (0)*

*P<0.05, **P<0.01
ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blockers; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro-brain natriuretic peptide; NYHA, New York Heart Association; TnT, Troponin T.

of circulatory congestion.

However, N-terminal pro-brain natriuretic peptide (NT-proBNP) represents the N-terminal fragment of pro-BNP, the high molecular weight precursor of biologically active BNP. N-terminal pro-brain natriuretic peptide has a relatively long half-life and is stable in whole blood. Concentrations of NT pro-BNP are increased in patients with CHF and correlate with prognosis![11–13] Since CHF is a complex clinical syndrome, a single biomarker may not reflect all of its characteristics. Theoretically, cTnT is a marker of myocyte injury while NT-proBNP reflects cardiac load.

This study examines the contribution of combined measurements of cTnT and NT-proBNP in patients with CHF in absence of acute coronary syndrome.

Methods

Subjects

The study population consisted of 71 consecutive patients admitted to our hospital between July 2001 and March 2002 for the management or evaluation of decompensated CHF. No patient had suffered a myocardial infarction or unstable angina pectoris within 3 months prior to hospitalisation, and no electrocardiographic changes or increase in creatine kinase (CK) were present upon admission. The criteria for a diagnosis of left heart decompensation on initial presentation used in this study were: (1) dyspnea or orthopnea requiring emergency hospitalisation, intravenous furosemide, and infusion of nitrates or inotropic agents, and (2) roentgenographically apparent pulmonary oedema and presence of moist rales on auscultation. Patients with cancer and undergoing hemodialysis were excluded. The demographic and baseline clinical characteristics of the study population are presented in Table 1.

Serum cTnT and NT-proBNP were measured with commercially available immunoassay kits (Roche Diagnostics, Tokyo, Japan). All study procedures were in accordance with the ethical institutional guidelines of Kyoto University.

Long-Term Clinical Events

The subsequent incidence of adverse cardiac events was recorded until December 2002. Significant adverse cardiac events were defined as sudden death without apparent ischemia, death from CHF, or rehospitalisation of the patient for management of cardiac decompensation with pulmonary oedema. Information pertinent to a patient's death occurring outside the hospital between follow-up visits was obtained from the family.

Statistical Analysis

Data are expressed as mean±standard error. The study variables were compared by factorial analysis of variance for continuous variables. A receiver–operator characteristic (ROC) curve was used to determine the cut-off value of NT-proBNP which predicts cardiac decompensation and cardiac events. Adverse cardiac event-free rate, were constructed by Kaplan–Meier's method, log-rank test. A P value<0.05 was considered statistically significant.

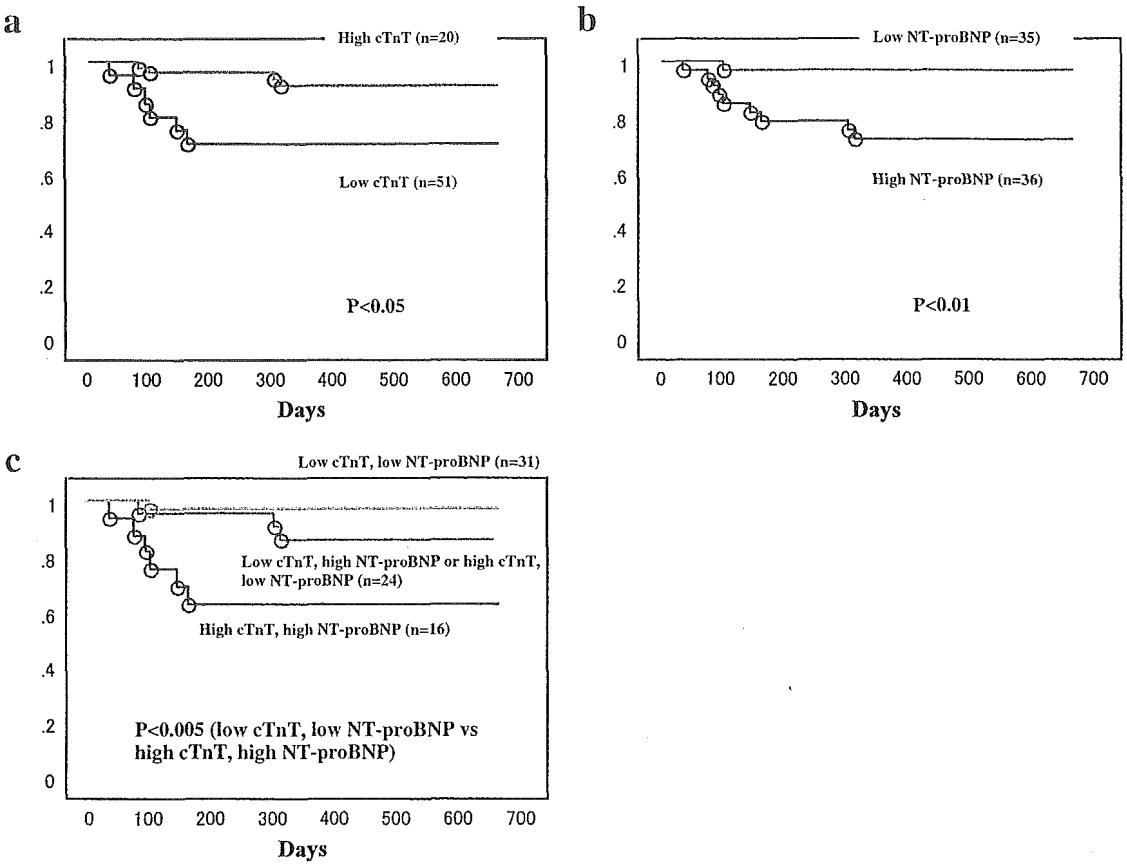

Fig 1. (a) Adverse cardiac event-free rate of patients with cTnT concentrations ≥0.01 ng/ml vs patients with cTnT concentrations <0.01 ng/ml. (b) Adverse cardiac event-free rate of patients with NT-proBNP concentrations ≥1,357 pg/ml vs patients with NT-proBNP concentrations <1,357 pg/ml. (c) Adverse cardiac event-free rate of patients with combined measurements of cTnT and NT-proBNP concentrations.

Results

Measurements of NT-ProBNP and TnT

The mean serum NT-proBNP concentration upon hospital admission of the 71 patients was 5,062±1,537 pg/ml (median=1,357 pg/ml). The mean NT-proBNP concentrations in patients of the NYHA functional class I, II, III, and IV were 954±361 (n=10), 1,673±473 (n=22), 2,902±771 (n=22), and 14,659±5,839 pg/ml (n=17), respectively. The cut-off value determined by ROC analysis for cardiac decompensation and cardiac events was 1,050 pg/ml (sensitivity 80%, specificity 67%) and 2,000 pg/ml (sensitivity 59%, specificity 67%), respectively. Age, CK concentration, and left ventricular ejection fraction and enddiastolic dimension measured echocardiographically did not correlate with NT-proBNP in this small population (data not shown).

The serum concentration of cTnT upon admission into the hospital was ≥0.01 ng/ml in 20 of the 71 patients (0.037±0.003 ng/ml). Cardiac troponin T was ≥0.01 ng/ml in 0/10 (0%), 6/22 (27%; 0.037±0.004 ng/ml), 7/22 (31%; 0.031±0.005 ng/ml) and 7/17 (41%; 0.046±0.008 ng/ml) patients in the NYHA functional classes I, II, III, and IV, respectively.

The mean serum concentration of NT-proBNP in the group of patients with high cTnT was significantly higher than in patients with low cTnT values (P<0.001). In contrast, age, CK and left ventricular ejection fraction were similar in both cTnT groups (Table 2). Comparisons between patients with and without cardiac decompensation are shown in Table 3. Concentrations of NT-proBNP in patients with cardiac decompensation were significantly higher than those in patients without (P<0.05).

Measurements of cTnT and NT-ProBNP, and Adverse Cardiac Events

Adverse cardiac events were observed in 10 patients (2 deaths from CHF and 8 cases of rehospitalisation for the management of cardiac decompensation with pulmonary oedema). The patients were divided into groups according to values of cTnT and NT-proBNP. The adverse cardiac event-free rate among the 20 patients with cTnT concentrations ≥0.01 ng/ml was significantly lower than the 51 patients with cTnT concentrations <0.01 ng/ml (P<0.05, Fig 1a). Similarly, the adverse cardiac event-free rate among the 36 patients with NT-proBNP concentrations ≥1,357 pg/ml was significantly lower than the 35 patients with NT-proBNP <1,357 pg/ml (P<0.01, Fig 1b). When groups were allocated according to both cTnT and NT-proBNP measurements, the 16 patients with high concentrations of both cTnT and NT-proBNP had a significantly lower adverse cardiac event-free rate than the 31 patients who had low cTnT and low NT-proBNP concentrations upon commencement of the study (P<0.005, Fig 1c).

Table 4 Hypothesis of Relationship Between Measurements of NT-ProBNP and TnT

	Low TnT	*High TnT*
Low NT-proBNP	*Without ongoing myocyte injury or myocardial load.*	*No myocardial load however, subclinical myocyte injury is ongoing. Patient is at risk of heart failure in the near future.*
High NT-proBNP	*Patient has heart failure without ongoing myocyte injury. Patient will stabilize with optimal treatment for heart failure.*	*Patient has heart failure with ongoing myocyte injury. If TnT concentrations do not decrease, heart failure may progress*

NT-proBNP, N-terminal pro-brain natriuretic peptide; TnT, Troponin T.

Discussion

In the present study, cTnT and NT-proBNP were reliable prognostic markers, both singly and in combination. Serum concentrations of cTnT≥0.01 ng/ml were considered significant[14] Assay of NT-proBNP is a new technology and normal values were reported approximately as 20 pg/ml[15,16] In our study, while mean NT-proBNP rose in the NYHA functional class, a similar correlation was not observed with mean cTnT concentrations. Moreover, 65% of patients with cardiac decompensation did not have a high serum cTnT concentration, and 15% had elevated concentrations despite being in a compensated state (Table 3). Troponin T seems to be a less sensitive marker of congestion.

We recently hypothesised that when managing heart failure, the therapeutic goals should be: (1) the relief of circulatory congestion and rapid lowering of markers of myocardial load, and (2) the mitigation of myocyte injury and lowering of markers of myocyte injury during long-term follow up[17] In this hypothesis, cTnT and NT-proBNP are important biochemical markers. The relationship between TnT and BNP and heart failure, based on our hypothesis, is shown in Table 4. These markers are easy to determine within a few hours and can be repeated for patient follow up, without inter-observer variability. In the future, the combination of these tests may be used in bedside clinical settings[18,19] Unfortunately, a multivariate analysis was not used to evaluate the prognostic value of these parameters because of our small sample numbers. Recently, Ishii et al reported that elevated cTnT and BNP on admission independently correlated with an increase in cardiac event rates in patients who were admitted to the coronary care unit for worsening chronic heart failure[14]

Although the mechanism of myocyte injury and the release of cTnT in CHF is not completely understood, cTnT seems to reflect ongoing myocyte injury even during compensated periods of CHF[7-10] Whether this indicates irreversible or reversible myocyte injury requires further investigation. The cytosolic pool for cTnT has been estimated at 6–8%. The release of protein may be because of a transient leak from the cytosol due to loss of sarcolemmal integrity during reversible ischemia, or from its continuous release when ischemic injury is irreversible[20,21]

No guidelines have been issued regarding the use of biochemical markers as part of the management of CHF. Recently, Maeda et al reported that BNP after optimized treatment for heart failure, rather than BNP before treatment, is an independent risk factor for morbidity and mortality in patients with congestive heart failure[22] We were unable to obtain follow up NT-proBNP data. While further studies are necessary, we anticipate that these assays will become the new monitoring standards in this patient population.

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Primary Cardiovascular Events and Serum Lipid Levels in Elderly Japanese with Hypercholesterolemia Undergoing 6-Year Simvastatin Treatment: A Subanalysis of the Japan Lipid Intervention Trial

Hisanori Horiuchi, MD, Toru Kita, MD, Hiroshi Mabuchi, MD, Masunori Matsuzaki, MD, Yuji Matsuzawa, MD, Noriaki Nakaya, MD, Shinichi Oikawa, MD, Yasushi Saito, MD, Jun Sasaki, MD, Kazuaki Shimamoto, MD, Hiroshige Itakura, MD

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Primary Cardiovascular Events and Serum Lipid Levels in Elderly Japanese with Hypercholesterolemia Undergoing 6-Year Simvastatin Treatment: A Subanalysis of the Japan Lipid Intervention Trial

Hisanori Horiuchi, MD,[*] Toru Kita, MD,[*] Hiroshi Mabuchi, MD,[†] Masunori Matsuzaki, MD,[‡] Yuji Matsuzawa, MD,[§] Noriaki Nakaya, MD,[‖] Shinichi Oikawa, MD,[¶] Yasushi Saito, MD,[#] Jun Sasaki, MD,[**] Kazuaki Shimamoto, MD,[††] Hiroshige Itakura, MD,[‡‡] and the J-LIT Study Group

OBJECTIVES: To determine the relationship between serum lipid levels and the incidence of coronary events in older Japanese hypercholesterolemic patients without prior coronary heart disease (CHD).

DESIGN: Post hoc subanalysis of the results in the Japan Lipid Intervention Trial.

SETTING: A large-scale cohort observational study conducted throughout Japan.

PARTICIPANTS: Men aged 35 to 70 and postmenopausal women younger than 70 with serum total cholesterol (TC) level of 220 mg/dL or greater treated for 6 years with low-dose simvastatin (52,421 total patients). After exclusion of 5,127 patients because of prior CHD and 4,934 patients because of incomplete data, 42,360 patients were divided into an older (9,860 patients, aged 65–70, mean age 67.1) and younger (32,500 patients, younger than 65, mean age 54.9) group and analyzed.

MEASUREMENTS: Fasting serum lipid levels were measured every 6 months. Major coronary events, including fatal or nonfatal myocardial infarction, and sudden cardiac death as the primary endpoint and other cardiovascular diseases, including onset of angina pectoris, cerebrovascular events, and any causes of death, as the secondary endpoints were monitored.

RESULTS: Simvastatin treatment in older patients was as safe and effective as in younger patients. Incident rates of major coronary events were 1.30 per 1,000 patient-years in the older group and 0.80 per 1,000 patient-years in the younger group. The incidence of a major coronary event was correlated to serum TC and low-density lipoprotein cholesterol (LDL-C) levels in both groups. The absolute risk of major coronary events in the older group was higher than in the younger group at any level of LDL-C, whereas the relative risk increased by 1.7% with an elevation of each 1 mg/dL LDL-C level in both groups. In the older group, the risk of major coronary events also increased as triglyceride level increased, whereas the risk decreased as high-density lipoprotein cholesterol level increased above 60 md/dL.

CONCLUSION: The LDL-C level-dependent increase of relative risk of CHD was similar in elderly and younger patients, whereas the absolute risk at any LDL-C level in elderly patients was higher than in younger patients. **J Am Geriatr Soc 52:1981–1987, 2004.**

Key words: serum cholesterol; coronary event; J-LIT study; elderly Japanese; simvastatin

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Controlling serum cholesterol levels is a rational strategy for prevention of coronary heart disease (CHD), as epidemiological and lipid intervention studies conducted in the United States and Europe have shown.[1–3] In those countries, CHD has been the main cause of death, and 21% to 24% of people have died of CHD,[4,5] whereas CHD accounts for merely 7% to 8% of deaths in Japanese living in Japan.[6] Despite this striking difference in CHD mortality between these populations, serum lipid levels have recently

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been similar in both populations.[7] The difference in mortality may be due to genetic backgrounds or environmental conditions, which are yet to be clarified.

The American guidelines for CHD prevention, Adult Treatment Panel III,[8] recommend that cholesterol levels should be controlled according to the individual absolute risk of CHD, estimated by the results of the Framingham study.[1] Nonetheless, the estimate of absolute risk of CHD in Japanese individuals is difficult, because no large-scale cohort studies have not been performed so far, only some small studies.[9–11] Recently, the dietary preferences of Japanese people have become progressively westernized, and their serum lipid profiles have been deteriorating rapidly.[12] It has currently become urgent in Japan to evaluate the relationship between lipid levels and the incidence of coronary events based on large-scale cohort studies, which provide fundamental data for preventive medicine. In addition, the elderly population in Japan has been rapidly increasing. In 2020, more than 30 million of 127 million people will be aged 65 and older.[6] Because cardiovascular events more frequently occur in the aged,[1,4,8] the incidence of CHD is likely to increase remarkably in Japan. Under these circumstances, the Japan Lipid Intervention Trial (J-LIT),[13–16] a large-scale observational cohort study in which many physicians throughout Japan participated, was conducted.

Subanalyses of lipid intervention studies with statins (3-hydroxy-3 methyl glutaryl coenzyme A reductase inhibitors) conducted in Western countries revealed that the treatment in older patients was as effective as that in younger patients for the prevention of CHD.[17–21] Recently, the Prospective Study of Pravastatin in the Elderly at Risk[22] conducted in Europe indicated that lipid-lowering therapy reduced CHD risk 21% in high-risk elderly subjects aged 75 to 82, but it was reported that increased serum cholesterol levels correlated with decreased mortality in patients aged 85 and older.[23] Furthermore, the Honolulu study[24] demonstrated that mortality was highest in the lowest quartile of total cholesterol (TC) level in Japanese Americans aged 72 to 92. Thus, lipid-lowering therapy, especially for elderly patients without prior CHD, should be well tuned to accomplish the goal.

To provide fundamental data about the relationship between serum cholesterol levels and CHD risk in elderly Japanese, the results of the J-LIT study were analyzed, focusing on patients aged 65 to 70 without a history of CHD.

METHODS

Study Design

The design of the J-LIT was described previously.[13] Briefly, men aged 35 to 70 and postmenopausal women younger than 70 with serum TC levels of 220 mg/dL or greater were enrolled. The exclusion criteria included recent myocardial infarction (MI) or stroke occurrence within a month, uncontrolled diabetes mellitus, serious complications of hepatic or renal disease, secondary hypercholesterolemia, malignant tumors, and illness with poor prognosis. More than 6,500 general practitioners throughout Japan treated patients with open-label simvastatin (5–10 mg/d) for 6 years during 1993–99. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald formula.

Serum lipid levels, drug-related adverse events, and clinical status were monitored every 6 months. The primary endpoint was a major coronary event, defined as fatal and nonfatal MI, and sudden cardiac death. The secondary endpoints were other cardiovascular diseases, including onset of angina pectoris; cerebrovascular accidents; and any other causes of death. The Endpoint Classification Committee of the study verified all coronary events and death. The Adverse Event Evaluation Committee evaluated the adverse drug events. Each patient was informed of the study purpose, drug efficacy, and need for long-term treatment. Of the 52,421 enrolled patients, 5,127 were excluded because of prior CHD (*International Classification of Disease* code I20 to I25 and prior coronary intervention), and 4,934 patients were excluded for the following reasons: violation of the protocol (n = 995), unwillingness to participate (n = 6), and incomplete data for covariates (n = 3,933). The remaining 42,360 patients were divided into two groups (aged 65–70: 9,860 patients, mean age 67.1; aged <65: 32,500 patients, mean age 54.9) and analyzed.

Statistical Analysis

All data were analyzed using the survival analysis method. The baseline lipid profiles and continuous variables were assessed using the paired or unpaired *t* test or chi-square test. For analysis of baseline characteristics determined using categorical outcomes and adverse drug events, the differences between groups were compared using the chi-square test. The incidence of the events was analyzed in relation to average lipid levels during the follow-up period, and the differences between groups were compared using log-rank test. The relative risk and its 95% confidence interval and incidence of the primary endpoint was calculated using the Cox proportional hazards model with adjustment for baseline characteristics such as sex, hypertension, diabetes mellitus, and smoking. For all statistical analysis, $P < .05$ was considered significant. All statistical calculations were performed using SAS software (version 6.12, SAS Institute, Inc., Cary, NC).

RESULTS

Baseline Characteristics

The baseline characteristics of the older and younger groups are shown in Table 1. Fewer men than women were enrolled in both groups. In addition, there was a smaller percentage of male patients in the older group than in the younger group (24.1% vs 35.1%). There were fewer smokers and alcohol consumers in the older group. Other baseline characteristics between the two groups were largely similar (Table 1). Most patients (97%) in both groups took simvastatin 5 mg/d. Other medications were nearly similar in both groups. The most frequently used drugs in both groups were calcium-channel blockers (31.4% in the older group and 21.4% in the younger group), angiotensin-converting enzyme inhibitors (13.0% and 12.4%, respectively), and beta-blockers (7.6% and 9.0%, respectively).

Of enrolled patients without prior CHD, 42,360 (91.5%) were followed, and 4,934 were excluded. No differences were observed in baseline characteristics, including mean age, sex ratio and serum TC level, between the

Table 1. Relative Risk (RR) of Major Coronary Events at Baseline

	Age			
	<65* (n = 32,500)		65–70† (n = 9,860)	
Risk Factor	%	RR (95% CI)	%	RR (95% CI)
Male	35.1	2.25 (1.51–3.34)	21.2	2.03 (1.17–3.52)
Obesity (body mass index >25 kg/m^2)	24.4	0.93 (0.66–1.33)	21.1	1.05 (0.62–1.75)
Hypertension	44.1	2.24 (1.58–3.17)	51.9	2.13 (1.27–3.55)
Diabetes mellitus	15.2	2.11 (1.46–3.06)	15.3	2.36 (1.41–3.95)
Electrocardiogram abnormality	12.0	1.87 (1.25–2.81)	16.4	1.70 (1.00–2.90)
Family history of coronary heart disease	5.1	2.67 (1.63–4.39)	3.5	2.57 (1.11–5.94)
Smoker	18.7	1.64 (1.10–2.45)	9.2	1.46 (0.73–2.89)
Alcohol drinker	32.4	0.63 (0.41–0.98)	17.7	0.63 (0.31–1.29)

Mean ± standard deviation = * 54.9 ± 6.7; † 67.1 ± 1.6 years old.
CI = confidence interval.

followed and excluded patients in the older group. In the younger group, slight differences between the followed and excluded patients were observed in proportion of men (39.2% vs 35.1%) and mean age (53.9 vs 54.9 years), whereas no difference was observed in TC level. It is unlikely that these differences in the younger group affect the results of this subanalysis.

Lipid Profiles

The baseline levels of TC, LDL-C, and high-density lipoprotein cholesterol (HDL-C) were similar between the two age groups, whereas triglyceride (TG) level was lower in the older group (Table 2). The mean baseline TC levels were 267 mg/dL in the older group and 271 mg/dL in the younger group. The mean reduction rates during the follow-up period under low-dose simvastatin treatment in the older and younger groups were 19.5% and 18.1% for TC, 28.2% and 26.2% for LDL-C, and 14.9% and 16.3% for TG, respectively. HDL-C level was 4.9% and 4.4% elevated, respectively (Table 2). The reduction in LDL-C level in both groups was similar at 6 months of treatment and continued throughout the follow-up period (data not shown). Thus, the lipid profiles of the two groups were similar at baseline and during treatment, indicating that simvastatin is as effective in older patients as in younger patients.

Drug-Related Adverse Events

Overall drug-related adverse events were observed in 3.18% of the older and 3.19% of the younger group ($P = .99$). The most frequently observed adverse events in both groups were hepatic dysfunction (0.99% in the older and 1.02% in the younger group, $P = .79$) and musculoskeletal disorders (0.81% and 0.90%, respectively, $P = .40$). No rhabdomyolysis occurred in either group,

Table 2. Patients' Lipid Profiles at Baseline and During Treatment

	Age	
	<65 (n = 32,500)	65–70 (n = 9,860)
Lipid Profile (mg/dL)	Mean ± Standard Deviation (% Change)	
Baseline		
TC	271 ± 36	267 ± 29
LDL-C	183 ± 34	181 ± 31
Triglyceride	202 ± 184	175 ± 110
HDL-C	52.8 ± 15.1	53.4 ± 15.1
During treatment		
TC	222 ± 30 (−18.1)*	215 ± 27 (−19.5)*
LDL-C	135 ± 30 (−26.2)*	130 ± 27 (−28.2)*
Triglyceride	169 ± 110 (−16.3)*	149 ± 68 (−14.9)*
HDL-C	55.1 ± 13.7 (+4.4)*	56.0 ± 13.7 (+4.9)*

* $P < .001$ baseline vs during treatment.
TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol.

although myopathy was reported in one patient in the older group and three patients in the younger group. The incidence of renal dysfunction was slightly higher in the older group (0.32%) than in the younger group (0.14%) ($P<.01$). None of differences in these adverse events were clinically significant. The incidence of other adverse events was not statistically significantly different between the two groups. It was concluded that simvastatin treatment in older patients aged 65 to 70 was as safe as in younger patients.

Coronary and Cerebrovascular Events and Death

Incidence rates during treatment of major coronary events, including fatal or nonfatal MI and sudden cardiac death, were 1.30 per 1,000 patient-years in the older group and 0.80 per 1,000 patient-years in the younger group ($P<.001$) (Table 3). Incidence rates of major coronary events in male patients were 2.45 per 1,000 patient-years in the older group and 1.41 per 1,000 patient-years in the younger group ($P=.003$). In female patients those rates were 1.00 and 0.47 per 1,000 patient-years, respectively ($P<.001$). Incidence rates of total coronary events, including major coronary events and newly developed angina pectoris, were 2.24 per 1,000 patient-years in the older group and 1.35 per 1,000 patient-years in the younger group ($P<.001$) (Table 3).

Incidence rates of ischemic cerebrovascular events, including cerebral thrombosis, cerebral infarction, transient ischemic attack, and reversible ischemic neurological deficit, were 2.61 per 1,000 patient-years in the older group and 1.29 per 1,000 patient-years in the younger group ($P<.001$) (Table 3). Thus, the incidence of major coronary events and ischemic cerebral accidents was higher in the older group.

Overall mortality was 2.4 times higher in the older group than in the younger group (Table 3). The proportions of cardiac deaths, death from malignancy, and death from other causes were similar in both age groups (Table 3).

Effects of Conventional CHD Risk Factors on the Development of Major Coronary Events

The contribution of the conventional risk factors for CHD to the development of major coronary events was analyzed (Table 1). Male sex, hypertension, diabetes mellitus, and family history of CHD were found to be significant risk factors of CHD in both age groups. Electrocardiogram abnormalities and smoking were statistically significant only for the younger group, possibly because of fewer patients enrolled in the older group. Body mass index of 25 kg/m^2 or greater did not increase the risk of CHD in either group in this study. Moderate alcohol consumption seemed to be a negative risk factor for CHD in both groups to a similar extent, although it was statistically significant only for the younger group. These conventional risk factors appear to contribute similarly in both age groups.

Incidence of Major Coronary Events and Lipid Profiles During the Follow-Up Period

The incidence rate of the major coronary events was analyzed in relation to serum lipid levels stratified by the average values during the follow-up period. As shown in Figures 1A and 1B, the incidence rate of major coronary events was higher in the older group than in the younger group at any level of serum TC and LDL-C. In both groups, major

Table 3. Death and Coronary and Cerebrovascular Events During Treatment

	<65 (n = 32,500)		65–70 (n = 9,860)		
Adverse Event	n	Incidence Rate†	n	Incidence Rate†	*P*-value*
Death, total	489	2.79	355	6.70	<.001
Cardiac	45	0.26	38	0.72	<.001
Noncardiac	444	2.53	317	5.98	<.001
Malignancy	185	1.06	132	2.49	<.001
Other	259	1.48	185	3.49	<.001
Coronary endpoint	236	1.35	119	2.24	<.001
Major coronary event	140	0.80	69	1.30	<.001
Myocardial infarction (fatal)	31	0.18	20	0.38	.007
Myocardial infarction (nonfatal)	105	0.60	42	0.79	.12
Sudden cardiac death	4	0.02	7	0.13	.001
Angina pectoris	96	0.55	50	0.94	.002
Cerebrovascular event	397	2.26	211	3.99	<.001
Ischemic cerebrovascular event	226	1.29	138	2.61	<.001
Cerebral thrombosis	120	0.68	66	1.25	<.001
Cerebral infarction	56	0.32	44	0.83	<.001
Transient ischemic attack, reversible ischemic neurological deficit	50	0.29	28	0.53	.008
Cerebral hemorrhage	83	0.47	31	0.59	.30
Subarachnoid hemorrhage	44	0.25	14	0.26	.86
Unclassified stroke	44	0.25	28	0.53	.002

* For log-rank test.
† Per 1,000 patient-years.

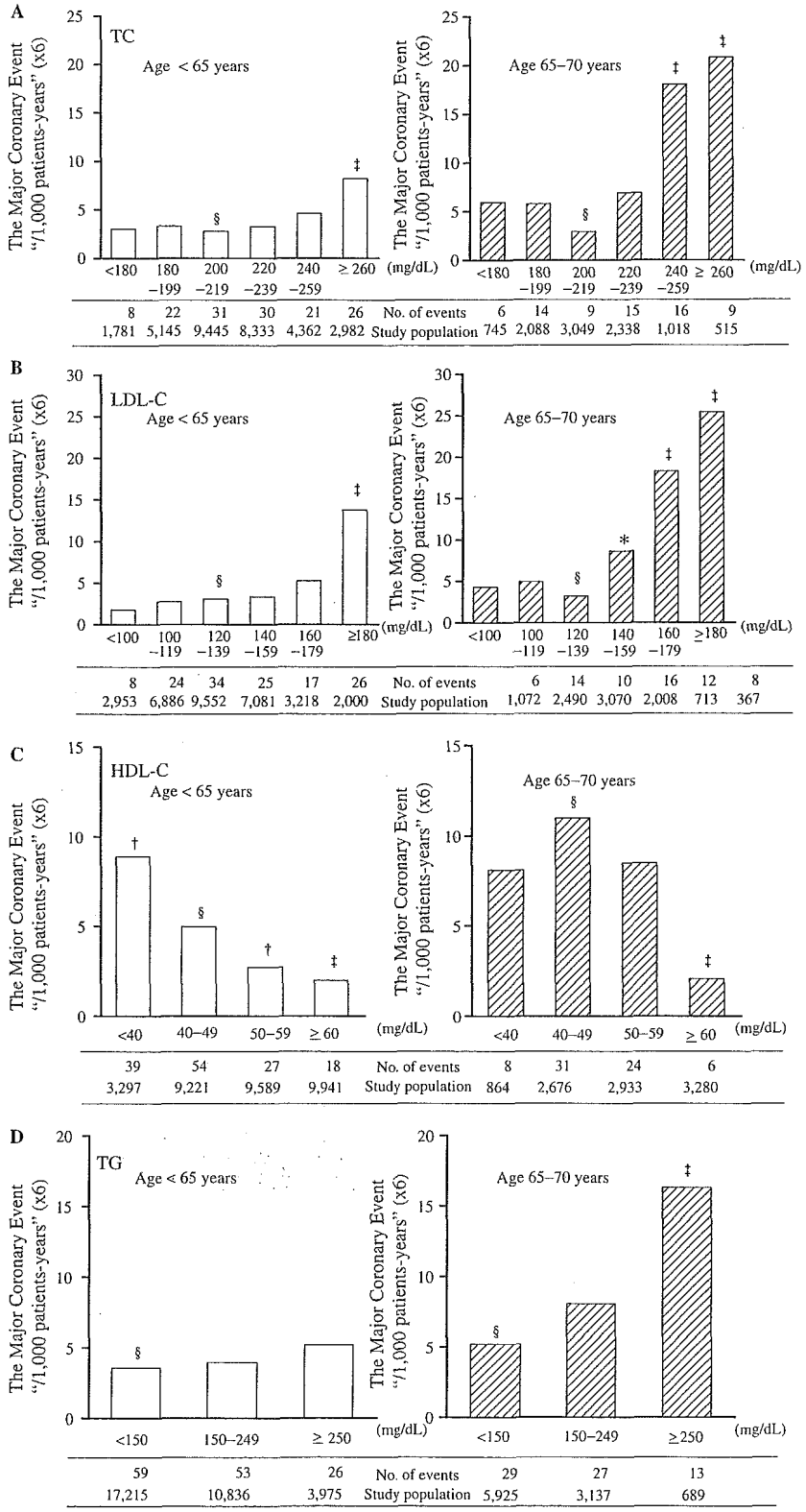

Figure 1. The relationship between the incidence of major coronary events and (A) serum total cholesterol TC, (B) low density lipoprotein-cholesterol LDL-C, (C) high density lipoprotein-cholesterol (HDL-C), and (D) triglyceride (TG) levels in the older and younger groups. The incidence rate of major coronary events was assessed using a Cox proportional hazards model according to stratified mean lipid levels during the follow-up period as indicated in the figures. These data were adjusted for sex, hypertension, diabetes mellitus, and smoking. $^{*}P<.05$; $^{\dagger}P<.01$; $^{\ddagger}P<.001$ versus reference category. Reference categories: TC = 200–219 mg/dL, LDL-C = 120–139 mg/dL, HDL-C = 40–49 mg/dL, and TG = <150 mg/dL.

coronary events increased as serum TC level elevated, and the increase was accentuated in patients with levels of 240 mg/dL or greater in the older group and 260 mg/dL or greater in the younger group (Figure 1A). Similarly, major coronary events increased in an LDL-C level–dependent manner in both groups, and the increase was accentuated in patients with levels of 140 mg/dL or greater in the older group and 180 mg/dL or greater in the younger group (Figure 1B). In a linear regression model, the incidence rate of major coronary events increased by 1.7% with an elevation of each 1 mg/dL LDL-C level in both age groups, although the absolute risk was higher in the older group (data not shown).

Major coronary events decreased as HDL-C level increased in the younger group, whereas in the older group major coronary events were higher in patients with levels lower than 60 mg/dL and declined abruptly in patients with levels of 60 mg/dL or greater (Figure 1C). Major coronary events in the younger group were not correlated with TG level. In the older group, major coronary events increased as TG level increased (Figure 1D).

DISCUSSION

The J-LIT study was the first successful large-scale prospective observational study in Japan. The overall results of the study clearly showed that the risk of CHD was positively correlated with LDL-C level and inversely correlated with HDL-C level in Japanese patients with hypercholesterolemia.[14,15] In this report, the J-LIT data of patients aged 65 to 70 without prior CHD were compared with those of patients aged 35 to 64. It was demonstrated that cholesterol-lowering treatment with simvastatin for older Japanese patients was as safe and effective as for the younger patients and that the absolute risk of CHD in older patients was approximately twice that of younger patients at any LDL-C level. Nonetheless, the LDL-dependent increase of the relative risk of CHD was similar.

Mean baseline TC (267 mg/dL) and LDL-C (181 mg/dL) levels in the older group decreased to 215 mg/dL (−19.5%) and 130 mg/dL (−28.2%), respectively, during the treatment. These values were similar to those in the younger group. In the J-LIT study, most patients (97%) took 5 mg/d of simvastatin. Why such a low dose of simvastatin reduced LDL-C levels by 28% in Japanese patients is not clearly understood.

The incidence of major coronary events, including fatal and nonfatal MI, and sudden cardiac death was 1.30 per 1,000 patient-years in the older group and 0.80 per 1,000 patient-years in the younger group. There were fewer men in both groups, possibly because more women than men are treated for hypercholesterolemia in Japan.[25] Additionally, there was a lower percentage of men in the older group than in the younger group (21.2 vs 35.1%). In male patients, the incidence rate of major coronary events was 2.45 per 1,000 patient-years in the older group and 1.41 per 1,000 patient-years in the younger group. In female patients, the incidence rate was 1.00 per 1,000 patient-years and 0.47 per 1,000 patient-years, respectively. The incidence rate of major coronary events in the older group was approximately twice as high in both sex subgroups.

The incidence rate of coronary events was potentially underestimated in the J-LIT study compared with that in the general Japanese population because all subjects were taking simvastatin, which might have direct antiatherosclerotic effects on coronary vessels[26,27] in addition to reducing lipids. In Western countries, statin treatment has been shown to reduce coronary events by 30% to 40% in primary[3,19] and secondary prevention studies.[2,17,20] If this reduction rate could be applied to the J-LIT results, the incidence of coronary events would be predicted to be approximately 1.4 to 1.6 times higher in the general Japanese population. Nevertheless, the incidence rate of coronary events in this study is much lower than in Western populations.[1,3,19] In the West of Scotland Coronary Prevention Study (WOSCOPS),[3] Scottish male hypercholesterolemic patients aged 45 to 64 (mean age 55) were followed with or without pravastatin treatment. In the pravastatin group, baseline TC level was 272 mg/dL and decreased by 20% during the follow-up period.[3] Mean age, baseline TC level, and reduction rate of TC of the pravastatin group were similar to those of the younger male group (mean age 54.9) in the J-LIT study. The incidence rate of coronary events was 11/1,000 patient-years for the pravastatin group in the WOSCOPS whereas the rate was 1.41/1,000 patient-years for the younger male group in the J-LIT study. Although there were differences in study conditions, the incidence of coronary events in Japanese male patients under statin treatment was one-eighth that of Scottish male patients.

The relative risk of major coronary events increased by 1.7% with an elevation of each 1 mg/dL in LDL-C level in both age groups, whereas the absolute risk at any level of TC and LDL-C was higher in the older group. The incidence rate of major coronary events markedly increased with TC level above 240 mg/dL and LDL-C level above 140 mg/dL in the older group. These TC and LDL-C levels were 20 and 40 mg/dL lower than those in the younger group, respectively. Generally, high-risk patients are good candidates for preventive medicines. In this viewpoint, elderly patients might receive more benefit from lipid-lowering therapy, but lipid intervention trials are required to establish the therapeutic benefits and strategies in elderly Japanese.

The preventive effect of HDL-C was also observed in the Japanese population. In the older group, the incidence of major coronary events decreased with HDL-C level above 60 mg/dL, whereas the incidence was HDL-C level–dependent in its wide range in the younger group. The role of TG level for the development of coronary events is controversial,[28] although the evidence is accumulating.[29] In the J-LIT study, TG level above 250 mg/dL was associated with a greater risk of major coronary events in the older patients, whereas no such relationship was observed in the younger group.

The incidence rate of ischemic cerebrovascular events, including cerebral thrombosis and infarction, transient ischemic attack, and reversible ischemic neurological deficit, was 2.61 per 1,000 patient-years in the older group and 1.29 per 1,000 patient-years in the younger group. The ratio of the incidence rate of ischemic cerebrovascular events (the older group/the younger group, 2.02) was larger than that of major coronary events (1.63), suggesting that aging may affect the occurrence of ischemic cerebrovascular events more strongly than of coronary events in the Japanese population.

In summary, the LDL-C level–dependent increase of the relative risk of CHD was similar in elderly and younger patients, whereas the absolute risk at any TC and LDL-C level in elderly patients was twice as high as in younger patients. Further lipid intervention trials would be required to establish the therapeutic benefits and strategies in elderly Japanese.

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Alterations in expression of angiopoietins and the Tie-2 receptor in the retina of streptozotocin induced diabetic rats

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Purpose: The angiopoietin (Ang)/Tie-2 system may play a role in vascular integrity and angiogenesis. In this study, we investigated alterations of the gene expression of Ang-1 and Ang-2 in the retinas of streptozotocin (STZ) induced diabetic rats.

Methods: In situ hybridization, reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analyses were performed to determine the mRNA and protein content for Ang-1 and Ang-2 and the Tie2 receptor in the retinas of STZ diabetic and age matched control rats.

Results: Using in situ hybridization analysis, Ang-1, Ang-2, and Tie2 mRNA expression was observed in the ganglion cell layer (GCL) and the inner nuclear layer (INL). While Ang-2 mRNA expression did not changed after 2 weeks, 1 month, or 3 months of STZ induced diabetes, it was increased in the GCL and slightly elevated in the INL after 6 months of diabetes. In contrast, Ang-1 and Tie2 mRNA expression was stable at every timepoint during 6 months of STZ induced diabetes. RT-PCR and western blot analyses confirmed the increase of Ang-2 expression after 6 months of diabetes. Furthermore, double staining of alpha-smooth muscle actin (αSMA) and Ang-2 mRNA demonstrated that the SMA positive cells surrounding Ang-2-expressing cells were decreased in the GCL.

Conclusions: Diabetes increases Ang-2 expression in the GCL accompanied by a reduction of αSMA positive perivascular cells. These changes may suggest a role for Ang-2 in the mechanism of pericyte loss in diabetic retinopathy.

In the retinas of both diabetic humans and diabetic animals, the degeneration and loss of pericytes are important features of morphological abnormality in the microvasculature of diabetic retinopathy [1-3]. Insufficient interaction between pericyte and vascular endothelial cells has recently been reported to cause a retinopathy that mimics diabetic retinopathy, including retinal edema and angiogenesis, hemorrhage, and retinal detachment [4]. This evidence suggests that either degeneration or direct loss of pericytes could contribute to most of the pathological changes observed in the later stages of diabetic retinopathy and is therefore an important phenomenon in understanding diabetic retinopathy. The mechanism of pericyte degeneration, however, is not well understood. High glucose has been reported to induce pericyte loss in vitro through several mechanisms, such as activation of sorbitol pathways [5], protein kinase C [6,7], and glycation end products [8,9]. In in vivo animal models of diabetes, inhibitors of these pathways have been shown to suppress pericyte loss in the retinal vasculature. More direct mechanisms such as activation of oxidative stress [10,11], nuclear factor-kappaB [12], and the proapoptotic effects of the Fas/Fas ligand system have also been suggested to play a role in pericyte loss [13].

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Angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2) are ligands for the Tie-2 receptor and bind with similar affinity [14,15]. Tie-2 is a member of the endothelium specific receptor tyrosine kinase families [16]. Ang-1 induces the auto-phosphorylation of Tie-2 in cultures of endothelial cells [15], whereas Ang-2 acts as an antagonist and inhibits Ang-1 induced phosphorylation of Tie-2 receptor in vascular endothelial cells [14]. The presence of Ang-2 destabilizes the vessels and it has been proposed that this is a necessary step for angiogenesis, whereas the presence of Ang-1 and the activation of Tie-2 stabilizes vessels. Tie-2-knockout mice die by day 9.5 to 10.5, due to immature vessels and lack of microvessel formation [17,18], although endothelial cell numbers are normal and tubular formation can be detected. A Tie-2 mutation in humans has been reported to cause venous malformations, which are typically an imbalance of endothelial cells and smooth muscle cells [19]. These findings suggest that the Ang and Tie-2 system are the key systems for the endothelial-stromal cell interaction during vascular development. Their role in diabetic retinopathy, however, remains unknown.

In the present study, the effect of diabetes on the retinal Ang/Tie-2 receptor system was investigated. Diabetes increased the gene expression of Ang-2 consistent with a role for this ligand in disruption of the vascular endothelium. However, Ang-1 and Tie-2 gene expression levels remained unchanged. The change in Ang-2 coincided with pericyte loss as determined by immunostaining for smooth muscle actin

(SMA). These data suggest that expression of the Tie-2 antagonist Ang-2 may induce pericyte loss in diabetic retinopathy.

METHODS

Animals: A rat model was used in which diabetes was induced by streptozotocin (STZ; Sigma Chemical, St. Louis, MO). Diabetes was induced in eight Sprague Dawly rats, each eight weeks old, by intravenous injection of 65 mg/kg of STZ in physiologic saline. We confirmed that the plasma glucose level in each rat was greater than 200 mg/dl 48 h later. Eight additional Sprague Dawly rats that were injected with an equal volume of saline alone served as nondiabetic control subjects. All rats were allowed free access to water and food before sacrifice. After injection of STZ, both non-diabetic control rats and diabetic rats were sacrificed and the eyes were enucleated at 2 weeks and at 1, 3, and 6 months. All procedures involving animal experimentation were conducted in accordance with both the guidelines for animal experiments of Kyoto University and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Histochemical samples: Four eye samples were obtained from each group. Briefly, after sacrifice of the rats using an anesthetic overdose, the eyes were obtained and enucleated. The eye samples were then fixed in 4% paraformaldehyde with phosphate buffered saline (PBS) at pH 7.4 for 2 h at 4 °C, dehydrated with a graded alcohol series, and then embedded in paraffin. For paraffin sections, the eyes were serially sectioned at a 5-μm thickness and placed on aminopropyltriethoxysilane coated glass slides (DAKO, Glostrup, Denmark) for in situ hybridization and immunohistochemical staining.

In situ hybridization: cDNA probes for human Ang-1 and Ang-2 were synthesized by reverse transcriptase-polymerase chain reaction (RT-PCR). For Ang-1 and Ang-2 cDNAs, a standard PCR was performed (PCR optimizer kit; Invitrogen, Vienna, Austria) using 5'-AGA ACC ACA CGG CTA CCA TGC T-3' (Ang-1 sense primer corresponding to nucleotides +671 to +692), 5'-TGT GTC CAT CAG CTC CAG TTG C-3' (Ang-1 antisense primer), 5'-AGC TGT GAT CTT GTC TTG GC-3' (Ang-2 sense primer corresponding to nucleotides +377 to +396), 5'-GTT CAA GTC TCG TGG TCT GA-3' (Ang-2 antisense primer corresponding to nucleotides +802 to +821), 5'-GCC TTA ATG AAC CAG CAC CAG G-3' (Tie-2 sense primer corresponding to nucleotides +335 to +356), and 5'-ACT TCT GGG CTT CAC ATC TCC G-3' (Tie-2 antisense primer corresponding to nucleotides +773 to +794) [20]. Sense oligonucleotide probes were used as negative controls. The probes were labeled using a DIG RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). Tissue sections (5 μm) were rapidly dewaxed, cleared with alcohol, rehydrated with PBS, pH 7.4, and then digested with Proteinase K (10 mg/mL; Sigma Chemical) for 7 min at 37 °C. The probes were applied in a formamide free diluent, and the slides were heated to 70 °C for 5 min and allowed to hybridize at 45 °C for 16 h. The sections were then washed twice with 2X SSC/50% formaldehyde buffer (1X SSC contains 150 mmol/L NaCl and 15 mmol/L trisodium citrate, pH 7.0) at 45 °C for 1 h and detected with alkaline phosphatase (AP) conjugated anti-digoxigenin antibody. After the hybridization products were washed once in AP chromogen buffer at room temperature, they were visualized with NBT/BCIP (Boehringer Mannheim). The slides were air dried and a coverslip was applied for microscopic examination.

Table 1. Total body weight and blood glucose level comparisons between diabetic (STZ) and control rats

Parameter	Group	2 weeks	4 weeks	3 months	6 months
Body weight (g)	Control	377.6±13.5	396.2± 2.0	414.9±17.1	437.7±22.2
	STZ	265.3± 2.5*	266.7±28.9*	304.5±16.9*	353.6±20.0*
Blood glucose level (mg/dl)	Control	95.5±11.0	96 ± 8.2	110 ± 3.3	85 ± 5.6
	STZ	279 ± 0.8*	330 ±55.5	304 ±15.9*	284.5±39.5*

Values shown are means±standard deviations. An asterisk ("*") indicates $p<0.05$ for the comparison between the control and diabetic rats.

Immunohistochemistry: To observe relationships between pericyte loss and Ang-2, immunohistochemical staining was performed with samples expressing Ang-2 mRNA via in situ hybridization. Sections used for in situ hybridization, were rinsed with PBS. A 0.3% hydrogen peroxide-methanol solution was applied to each specimen for 10 min to block endogenous peroxide activity. After incubating with blocking serum for 20 min, the specimens were incubated overnight at 4 °C with one of the primary antibodies: mouse monoclonal anti-α SMA, 1: 50 dilution (DAKO). Specimens were then washed for 10 min with PBS. A standard indirect immunoperoxidase protocol using the VECTASTAIN Elite ABC kit (Vector Laboratories, Burlingame, CA) was performed with 3-amino-9-ethylcarbazole (AEC; DAKO) as the substrate and all incubation steps were performed in a moist chamber. Finally, the slides were washed for 30 min with PBS, and a coverslip was applied with VECTASHIELD (Vector Laboratories) for viewing. As a negative control, normal mouse IgG (DAKO) was used as the primary antibody. Other staining procedures were the same as described earlier.

Semiquantitative reverse transcriptase polymerase chain reaction: Both non-diabetic control rats and diabetic rats were sacrificed and the eyes were enucleated at 2 weeks and at 1, 3, and 6 months. Retinal total RNA was collected by the acid guanidium thiocyanate-phenol chloroform extraction method, as described previously [21]. The primer sequences used were as follows: Ang-1, forward, 5'-CAG CAT CTG GA(A/G) CA(T/C) GT(A/G/T/C) ATG-3'; reverse, 5'-TTC (T/C)TT GTG TTT (A/G/T/C)CC (T/C)TC CAT-3'; Ang-2, forward, 5'-GT(T/G) GA(T/C) TT(T/C) CAG AG(A/G/T/C) AC(A/G/T/C) TGG-3'; reverse, 5'-CGA (A/G)TA GCC (T/G)GA (A/G/T/C)CC (T/C)TT CCA-3' [22]. Normalization of each cDNA concentration was performed using primers for β-actin, 5'-AGC TGA GAG GGAAAT CGT GC-3' (forward) and 5'-ACC AGA CAG CAC TGT GTT GG-3' (reverse) [23]. For RT-PCR, total cellular RNA, 2 μg from non-diabetic and diabetic rats were reverse-transcribed using an RNA PCR Kit, AMV (Takara, Kyoto, Japan). 5% of each reverse transcriptase product was amplified in the PCR reaction using the oligonucle-

otides described above. Polymerase chain reaction cycles were as follows: 95 °C, 3 min (once); 95 °C, 30 s; 55 °C, 1 min; and 72 °C, 45 s (25 cycles). RT-PCR products (about 372 bp) amplified with degenerate Ang-1 oligonucleotides from rat or RT-PCR products (about 453 bp) amplified with degenerate Ang-2 oligonucleotides from rat were then separated by 2% agarose gel electrophoresis. To investigate relative levels of Ang-1 and Ang-2 gene expression, semiquantitative analysis was then performed by measurement of the optical densities of the band with a fluorescence imager (FluorImager SI; Molecular Dynamics, Sunnyvale, CA) and its associated software WinRoof (Mitani Shoji, Fukui, Japan). The relative levels of mRNA expression were then calculated.

Real-time PCR: Total retinal RNA was extracted from 6 month old diabetic and control rats using RNAqueous-4PCR (Ambion, Austin, TX). First-strand cDNA was reverse transcribed from the total RNA using the First-Strand cDNA synthesis kit (Roche Pharmaceuticals, Mannheim, Germany) utilizing random hexamer nucleotides for initiation priming. Real-time PCR was performed using an ABI Prism 7700 PCR machine (Applied Biosystems, Foster City, CA). Primers and probes for rat VEGF was designed using Primer Express Soft-

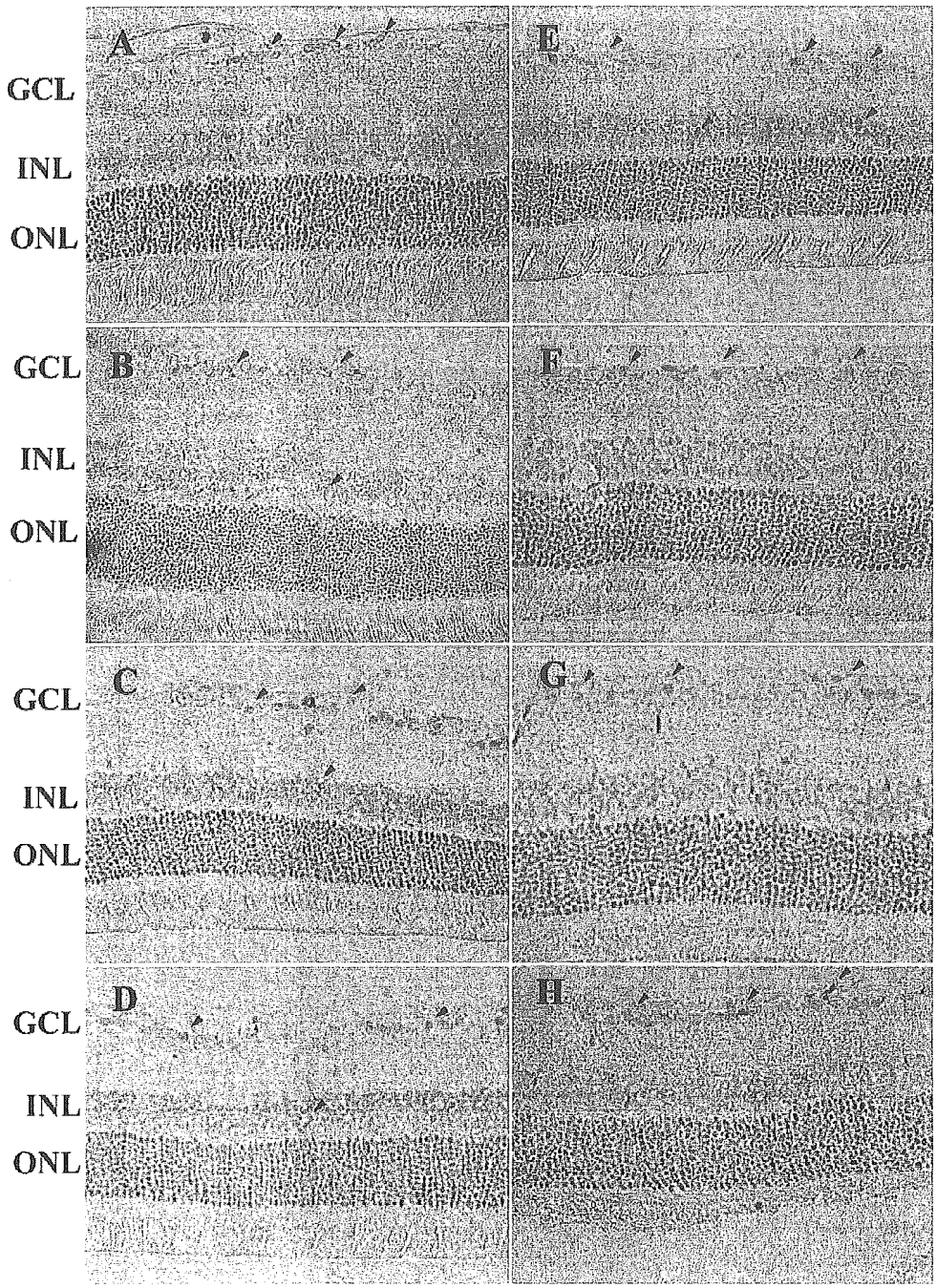

Figure 1. In situ hybridization analysis of Ang-1 during the development of diabetes. **A-D**: the retinas of STZ induced diabetic rats. **E-H**: the retinas of saline injected non-diabetic control rats. **A,E**: 2 weeks after injection. **B,F**: 1 month later after injection, **C,G**: 3 months after injection. **D,H**: 6 months later after injection. Weak Ang-1 mRNA expression was observed in the GCL and the INL (arrow heads). The intensity of Ang-1 mRNA expression was unchanged between diabetic and non-diabetic rats from 2 weeks to 6 months following STZ injection.

ware (version 2.0; Applied Biosystems). The real-time PCR cycle parameters were 48 °C for 30 min and 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. The relative differences were determined using the CT method as outlined in the Applied Biosystems protocol. The starting mRNA copy number of the target sequence was established by determining the fractional PCR threshold cycle (CT) number at which a fluorescence signal generated during the replication process passed above a threshold value. The initial amount of target mRNA in each sample was estimated from the experimental CT value with a standard curve.

Western blot analysis: Detergent soluble lysates of retina were prepared as previously described [24]. Briefly, retinas were collected from both 6 months diabetic and age matched control rats and extracted separately with ice cold lysis buffer (50 mM Hepes, pH 7.4, 10 mM EDTA, 100 mM NaF (Sigma Chemical), 10 mM sodium pyrophosphate (Sigma Chemical), 1% Triton X-100, 10 mM Na_3VO_4 (Sigma Chemical), 20 μM leupeptin (Sigma Chemical), 1.5 μM aprotinin (Sigma Chemical), and 2 mM PMSF (Sigma Chemical)) for 30 min. Lysates were cleared by centrifugation at 12,000 rpm for 15 min at 4 °C, and supernatants were removed and diluted with an equal

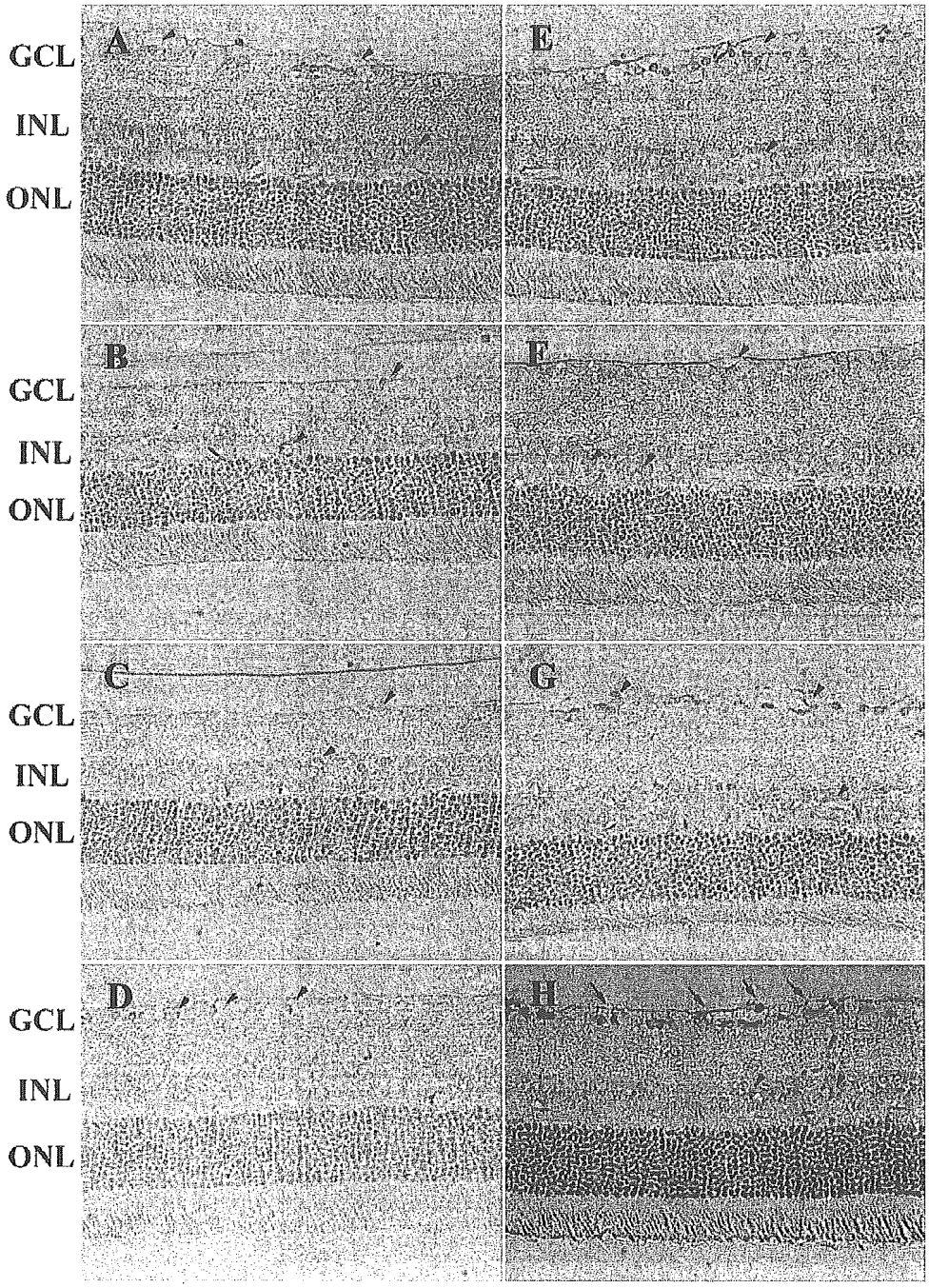

Figure 2. In situ hybridization analysis of Ang-2 during the development of diabetes. **A-D**: the retinas of STZ induced diabetic rats. **E-H**: the retinas of saline injected non-diabetic control rats. **A,E**: 2 weeks after injection. **B,F**: 1 month after injection, **C,G**: 3 months after injection. **D,H**: 6 months after injection. Weak Ang-2 mRNA expression was observed in the GCL and the INL at 2 weeks, 4 weeks and 3 months after STZ injection (arrow heads). The intensity of Ang-2 mRNA expression increased in the retina of diabetic rats at 6 months after STZ injection (arrows).

volume of 2% SDS sample buffer. Protein concentrations of the supernatants were determined with the bicinchoninic acid reagent (Pierce, Rockford, IL). Lysates were heated to 95 °C for 2 min, and equal volumes were subjected to SDS-PAGE under reducing conditions. To assay for Ang-1, Ang-2 and Tie-2, blots were incubated with polyclonal anti-Ang-1, Ang-2 and Tie-2 specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Lane loading was normalized by reblotting with a monoclonal anti-β-actin antibody (Sigma Chemical). Immunoblots were visualized using an enhanced chemiluminescence detection system (ECL, Amersham Biosciences, Piscataway, NJ) according to the instructions of the manufacturer.

Statistical analysis: Determinations for RT-PCR were performed in triplicate and results are expressed as means±standard error of the mean Student's t-test was used; 0.05 was selected as the α level.

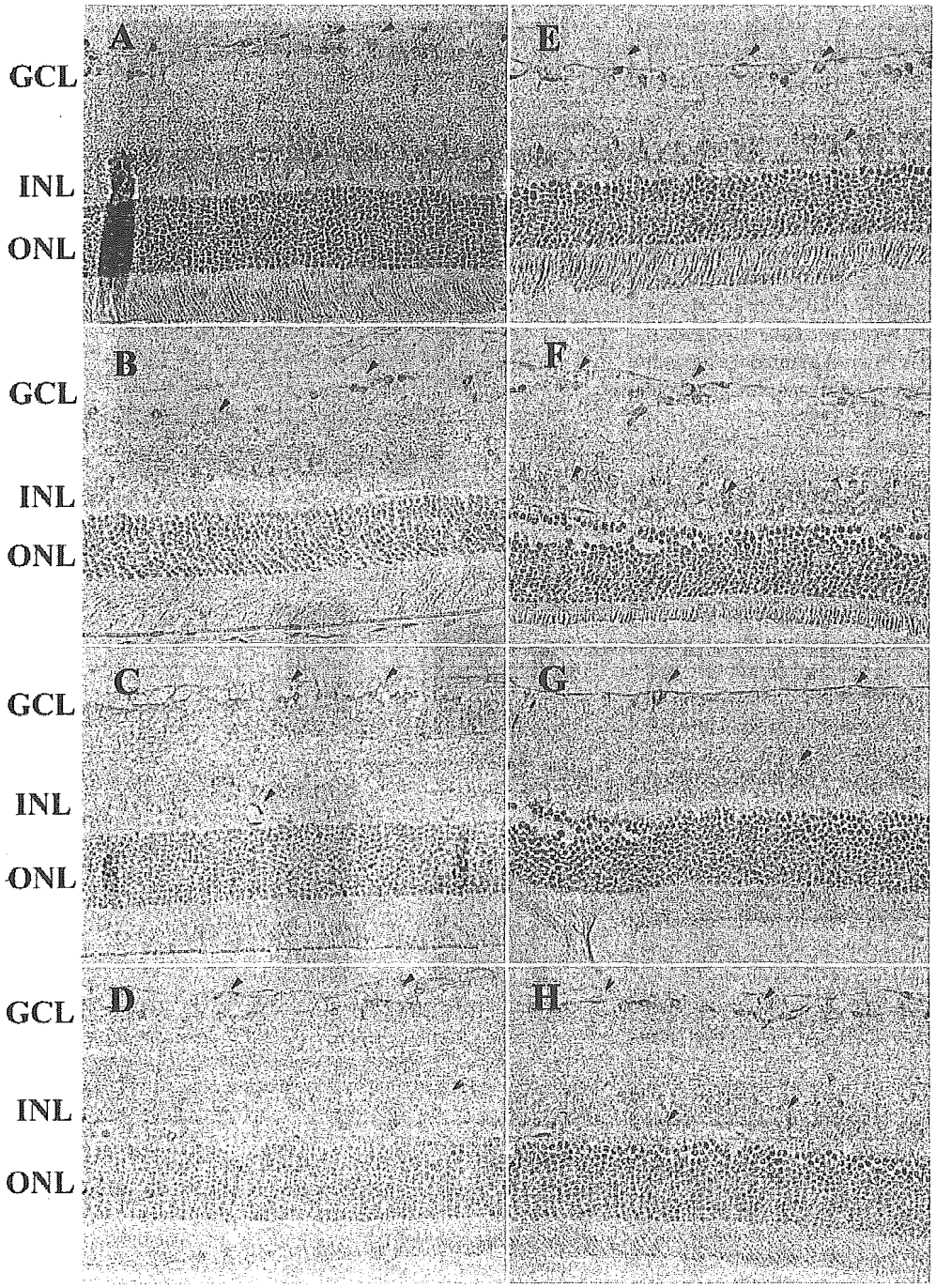

Figure 3. In situ hybridization analysis of Tie-2 during the development of diabetes. **A-D**: the retinas of STZ induced diabetic rats. **E-H**: the retinas of saline injected non-diabetic control rats. **A,E**: 2 weeks after injection. **B,F**: 1 month after injection, **C,G**: 3 months after injection. **D,H**: 6 months after injection. Tie-2 mRNA expression were observed in the GCL and the INL (arrow head). The intensity of Tie-2 mRNA expression was unchanged between diabetic and non-diabetic rats from 2 weeks to 6 months following STZ injection.