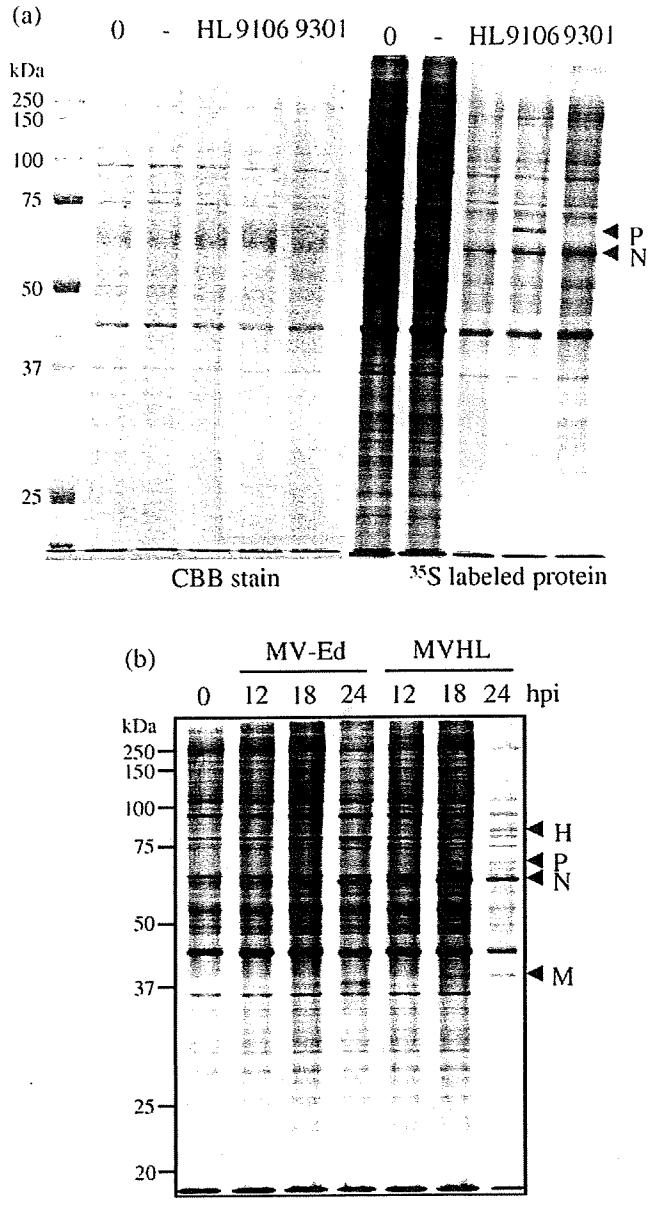

Fig. 2. The shut-off effect of other strain of MeV (a) Protein synthesis at 24 hpi in B95a cells infected with field isolates of MeV was examined similar to Fig. 1a (right panel) (0: 0 hpi; - :mock-infected B95a cells). To confirm the equivalence of protein, the autoradiograph gel was stained with Coomassie brilliant blue (left panel). (b) The protein synthesis in MeV-Ed- or MeV-HL-infected B95a cells was examined similar to Fig. 1a.

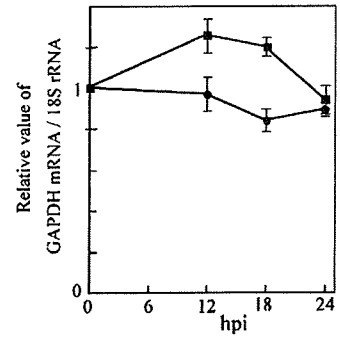

Fig. 3. The mRNA levels of GAPDH in B95a cells infected with MeV-HL. The expression levels of GAPDH mRNA in mock- (closed circle) or MeV-HL- (closed square) infected B95a cells were determined using one-step real-time RT-PCR and shown as means of three experiments.

mRNA to 18S rRNA was not suppressed by MeV-HL infection. Since the transcription level of GAPDH did not decrease during the period of shut-off, the inhibition of host protein synthesis in MeV-HL-infected B95a cells is suggested to occur at a post-transcription stage.

3.3. Modification of eIF4G, eIF4E and 4E-BP1 by MeV-HL infection

Previous reports on other viruses indicated that cap-binding proteins such as eIF4G, eIF4E, and 4E-BP1 are major targets for the virus-induced shut-off of host protein synthesis [6]. Picornavirus (except for cardiovirus) cleaved the eIF4G, which is one of the subunits of cap-binding complex eIF4F, by viral protease, $2A^{pro}$ [15–17]. This cleavage results in inhibition of binding of eIF4F to cap of host mRNA. In adenovirus- or influenza virus-infected cells dephosphorylation of eIF4E, which is a cap-binding protein, is observed [18–20]. Phosphorylation of eIF4E increases its affinity for the cap of mRNA [21]. Therefore, dephosphorylation of eIF4E by viral infection results in decrease of the affinity for the cap and may inhibit cap-dependent translation. The eIF4E is also regulated by eIF4E-binding protein-1 (4E-BP1). Encephalomyocarditis virus (EMCV), poliovirus and VSV dephosphorylate 4E-BP1 [22,23]. Dephosphorylated 4E-BP1 binds to eIF4E strongly, resulting in the suppression of cap-dependent translation. Considering these functions of cap-binding proteins, we first examined the characteristics of these three proteins in B95a cells at intervals after inoculation with MeV-HL. As shown in Fig. 4a, eIF4G was not cleaved throughout the course of MeV-HL-infection. Moreover, dephosphorylation was not observed for eIF4E and 4E-BP1 until 36 hpi (Fig. 4b and c). These results indicate that eIF4G and eIF4E are not involved in MeV-HL-induced shut-off of host protein synthesis as their function appear to be intact.

3.4. Accumulation of phosphorylated eIF2α in MeV-HL-infected B95a cells

Given that the modification of eIF4F was not detected in MeV-HL-infected B95a cells, we then focused on phosphorylation of eIF2α. It was reported that the interferon-inducible

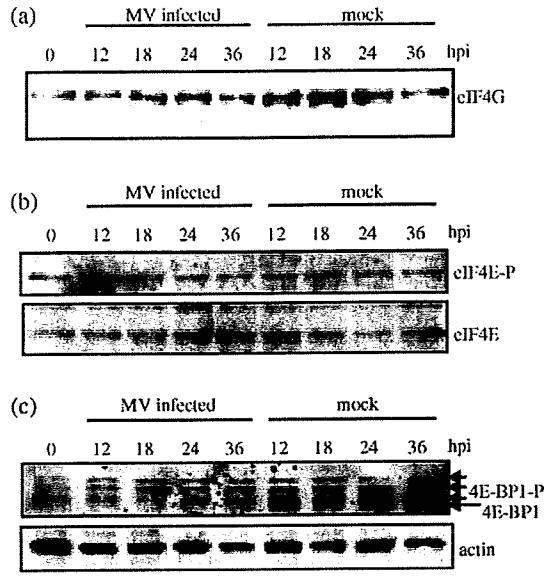

Fig. 4. Modification of the components of the eIF4F complex in MeV-HL-infected B95a cells. Expression levels of eIF4G and the phosphorylated states of eIF4E and 4E-BP1 in mock- or MeV-HL-infected B95a cells were determined by Western blotting assay. (a) Cell lysates were subjected to 6% SDS-PAGE and the proteins were transferred onto nitrocellulose membrane. The eIF4G was detected by Western blotting assay using rabbit antibody against eIF4G. (b) Detection of phosphorylated eIF4E by Western blotting assay using antibodies against phospho-eIF4E at serine 209 (upper panel) or eIF4E (lower panel) antibody. (c) The phosphorylation state of 4E-BP1 in the mock- or MeV-HL-infected B95a cells was examined by Western blotting assay using goat antibody against 4E-BP1. The quantity of protein was normalized to that of β-actin determined by goat antibody against β-actin.

PKR, known as a kinase that phosphorylate eIF2α at serine 51 [24], is activated by dsRNA during the infection with RNA viruses and involved as a host defense in preventing the translation of viral transcripts, concomitantly with the inhibition of host mRNA translation [25]. Considering such function of eIF2α, we analyzed the phosphorylation state of eIF2α by Western blotting assay with an antibody against phospho-eIF2α or eIF2α (Fig. 5). The ratio of phospho-eIF2α in MeV-HL-infected B95a cells increased after 12 hpi and reached a maximum (3.9-fold increase) at 18 hpi, although the effect was lower than that observed in the control with thapsigargin that induces eIF2α phosphorylation through ER-stress [26]. Thereafter, the ratio was sustained until 36 hpi. Phosphorylation of eIF2α occurred at a relatively early stage of infection, prior to the clear inhibition of host protein synthesis. The acceleration of host shut-off was accompanied by an increase in phosphorylation of eIF2α.

3.5. Suppression of MeV-HL-induced phosphorylation of eIF2α in B95a cells stably expressing S51A mutated human eIF2α

Involvement of phosphorylation of eIF2α in shut-off of host protein synthesis in MeV-HL-infected B95a cells was examined using B95a cells that stably express

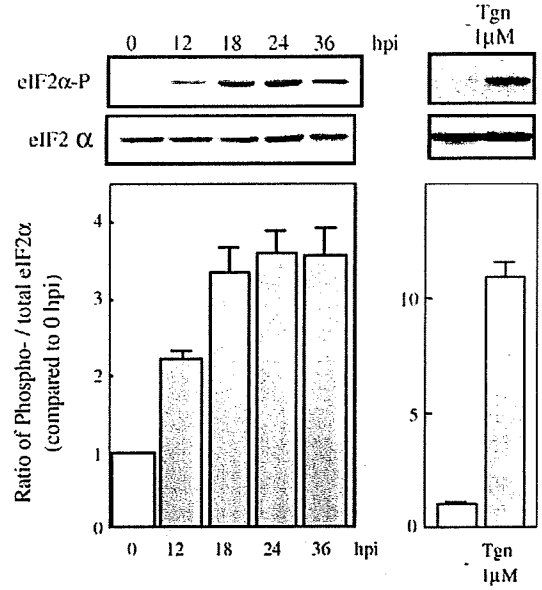

Fig. 5. Phosphorylation of eIF2α in MeV-HL-infected B95a cells. Lysates of mock- or MeV-HL-infected B95a cells were analyzed by Western blotting assay using antibodies against eIF2α-P (phosphorylated form at serine 51) or eIF2α on the same membrane (left, top). Quantitation of the relative amounts of phospho-eIF2α and the total eIF2α was measured and the ratio of phosphorylated eIF2α to total eIF2α (vs. 0 hpi) is shown as a bar graph (left, bottom). As a control experiment, B95a cells were treated with 1 μM thapsigargin for 1 h and shown as the same way as left column (right). The values are means ± standard errors of triplicate determinations.

eIF2α mutant, of which phosphorylation site serine 51, was replaced to alanine (B95a-2αS51A) and is able to inhibit the phosphorylation of endogenous eIF2α [27]. As a control experiment, the B95a cells that stably express wild type of eIF2α (B95a-2αWT) were used. The phosphorylation rate of total eIF2α in B95a-2αWT cells apparently increased at 18 hpi (Fig. 6a), whereas that in B95a-2αS51A cells was significantly inhibited. Shut-off of host protein synthesis was noted from 12 hpi in B95a-2αWT cells similar to the parental B95a cells. In B95a-2αS51A cells, shut-off of host protein synthesis was suppressed until 18 hpi (Fig. 6b and c) and the rate of host protein synthesis was higher than that of B95a-2αWT cells throughout the test period. These results indicate that the phosphorylation of eIF2α involved in shut-off of host protein synthesis in MeV-HL-infected B95a cells.

4. Discussion

In the present study, we showed that MeV-HL induces the shut-off of host protein synthesis in B95a cells. This shut-off is not specific feature of MeV-HL because other field isolates, 9106 and 9301 strain, also induce the shut-off in B95a cells. On the other hand, MeV-Ed that has been reported not to induce the shut-off in epithelial or epithelial-like cells did not induce the shut-off of host protein synthesis in B95a cells as well. Therefore, the inability of MeV-Ed to induce shut-off is suggested to be a characteristic of this strain

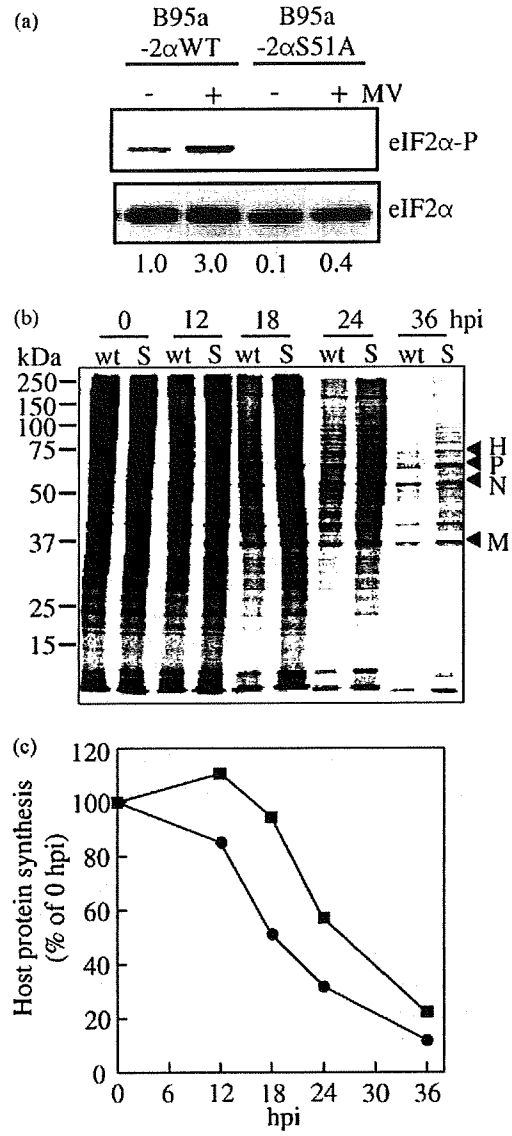

Fig. 6. MeV-HL-induced shut-off in eIF2αWT and S51A expressing cells. (a) eIF2α was detected by Western blotting assay using antibodies against phospho-eIF2α (upper panel), or eIF2α (lower panel) on the same membrane. The ratio of phosphorylated eIF2α (vs. mock infected B95a-2αWT) is shown under each lane. (b) Protein synthesis in MeV-HL-infected B95a-2αWT (wt) and B95a-2αS51A (S) cells was examined similar to Fig. 1a. Viral proteins are indicated to the right of the image. (c) The rates of host protein synthesis in B95a-2αWT cells (closed circle) or B95a-2αS51A (closed square) were determined from Fig. 6b by quantitation similar to Fig. 1b.

and independent of cell type. Similarly to MeV, Smith et al. reported that ability of reovirus to induce the shut-off of host protein synthesis is dependent of the viral strain [28].

The shut-off of host protein synthesis by virus infection was reported to be caused by a number of mechanisms such as inhibition of transcription, degradation of host mRNA and inhibition of translation. As the level of GAPDH mRNA was unaltered in MeV-HL-infected B95a cells, the shut-off by MeV-HL is suggested to be caused by inhibition of translation.

The shut-off of host translation is caused mainly by inhibition of the cap-dependent mechanism [6]. Contrary to many other virus-infected cells in which the components of the eIF4F complex including eIF4G, eIF4E and 4E-BP1 are involved in cap-dependent translation, they were not modified by MeV-HL infection. Therefore, the cap-binding activity of eIF4F complex appears to be intact. Instead, phosphorylation of eIF2α in MeV-HL-infected B95a cells was noted (Fig. 5). The phosphorylation rate of eIF2α correlated with the inhibition of host protein synthesis after MV infection. In addition, in B95a-2αS51A cells that stably expressed the eIF2α-S51A mutant, the shut-off phenomenon appeared to be suppressed compared with those in B95a and B95a-2αWT cells (Fig. 6). Therefore, phosphorylation of eIF2α is suggested as one of the mechanisms particularly at the early stage for the induction of host shut-off by MeV-HL infection.

Conner and Lyles reported that phosphorylation of eIF2α in VSV-infected cells suppressed viral translation rather than host translation [22]. In the case of MeV-HL infection, the suppression effect on host proteins was obviously much greater than that on viral proteins (Figs. 1a and 6b). MeV-HL mRNA may be more resistant to the effect of phosphorylated eIF2α than cellular mRNA. The mechanisms of the selective synthesis of viral protein in the shutoff stage of MeV-HL-infected cells are currently under investigation.

Recently, we also reported that the N protein of MeV-HL inhibits host translation by the binding to eIF3-p40 [13]. In our report, the inhibitory effect of the N protein is partial and inhibitory rate reaches a plateau at approximately 50–60%. On the other hand, MeV-HL-infection suppressed about 90% of the host translation (Fig. 1b). Experiment using eIF2αS51A mutant in this study, in which the inhibition of eIF2α phosphorylation observed in18 hpi lasted 24 hpi (data not shown) showed that the shut-off was inhibited at 18 hpi but became partial after 24 hpi (Fig. 6c). The expression level of the N protein increases rapidly after 18 hpi and reaches a peak at 24 hpi (data not shown). Taken together, we hypothesize that in MeV-HL-infected B95a cells the accumulation of phosphorylated eIF2α probably resulting from the replication of viral genome occurs at a relatively early stage of infection initiating the shut-off and then binding of increased N protein binds to eIF3-p40 and enhance the shut-off of host translation at later stage of infection.

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C型肝炎ウイルスとBリンパ腫

熊本大学大学院医学薬学研究部
感染症阻止学寄附講座特任教授
小原恭子
南カルフォルニア大学
ウイルス学講座助教授
町田圭吾
＊(財)東京都医学研究機構
東京都臨床医学総合研究所
感染症プロジェクトプロジェクトリーダー
小原道法

はじめに

C型肝炎ウイルス(HCV)は慢性肝炎，肝硬変，肝癌を引き起こすことが知られているが肝臓以外の臓器や組織で起きる病変にも深く関与していることが明らかとなってきている．HCV感染者のうち40～70%もの人が肝臓以外の疾患，いわゆる肝外病変を発症しているとされ[1]，典型的な肝外病変としては非ホジキン型リンパ腫(NHL；B細胞リンパ腫)，クリオグロブリン血症，膜性増殖性腎症，シェーグレン症候群などがある．我々はこれら肝外病変のうちB細胞リンパ腫の発症機序に関してHCVトランスジェニックマウスを用いて解析した．

1．HCV持続発現マウス(CN2-IRF-1$^{-/-}$マウス)を用いた解析

これまでに我々はHCVの構造蛋白質(Core，E1，E2蛋白質)をCre/loxPシステムにより生後任意の時期に発現するトランスジェニックマウス(CN2)を樹立している[2, 3]．CN2マウスはcre-adenovirus感染により肝臓でHCV遺伝子を発現誘導できるが，炎症反応によりすぐにHCV発現細胞が排除されてしまう．そこで，CTLやNK活性を抑制するためIRF-1を欠損させたマウスと交配した．その結果生後500日以上ウイルス蛋白質の発現が持続することが明らかとなった(図1)．さらに興味深いことに，生後180日を経過するとリンパ腫を発症するマウスが見出された．生後500日を過ぎると50%以上のマウスがリンパ性増殖を形成し死亡した(図2)．

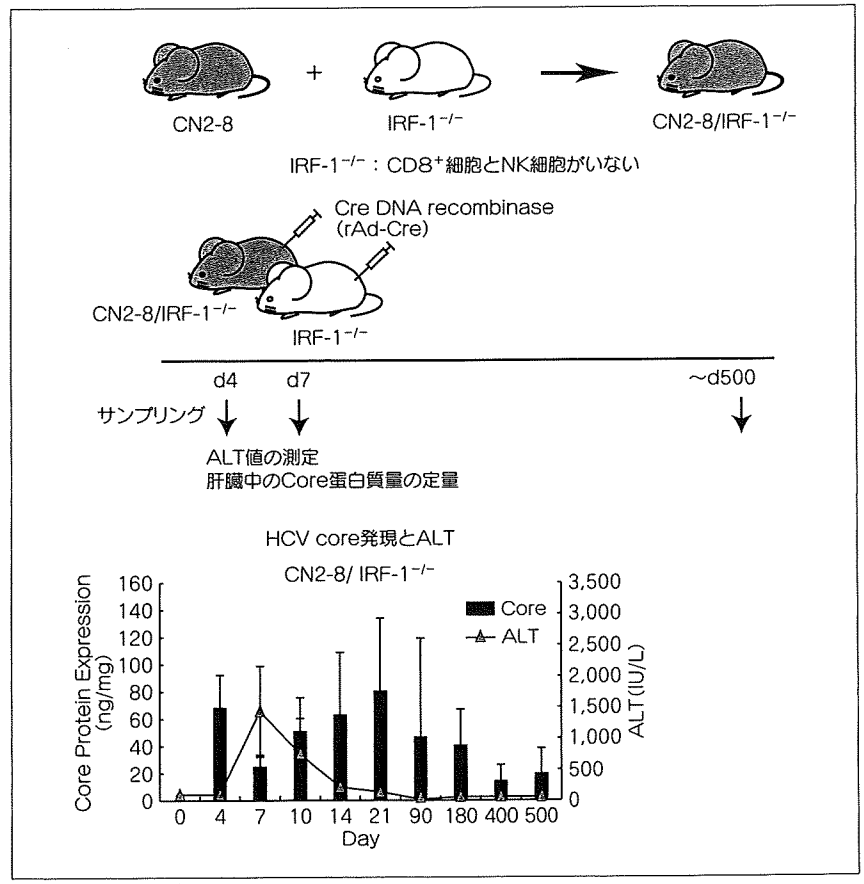

図1　CN2 IRF-1$^{-/-}$ マウス樹立(HCV持続発現)
Cre/loxPシステムでHCV遺伝子を導入したトランスジェニック(Cre/loxP/HCV-CN2)マウスと，IRF-1ノックアウトマウスを交配させることにより，任意の時期にHCV遺伝子をスイッチング発現し，HCV蛋白質を持続発現するCN2 IRF-1$^{-/-}$マウスを作製した．

＊〒156-0057　東京都世田谷区上北沢2丁目1番地6号
E-mail　kohara-mc@igakuken.or.jp

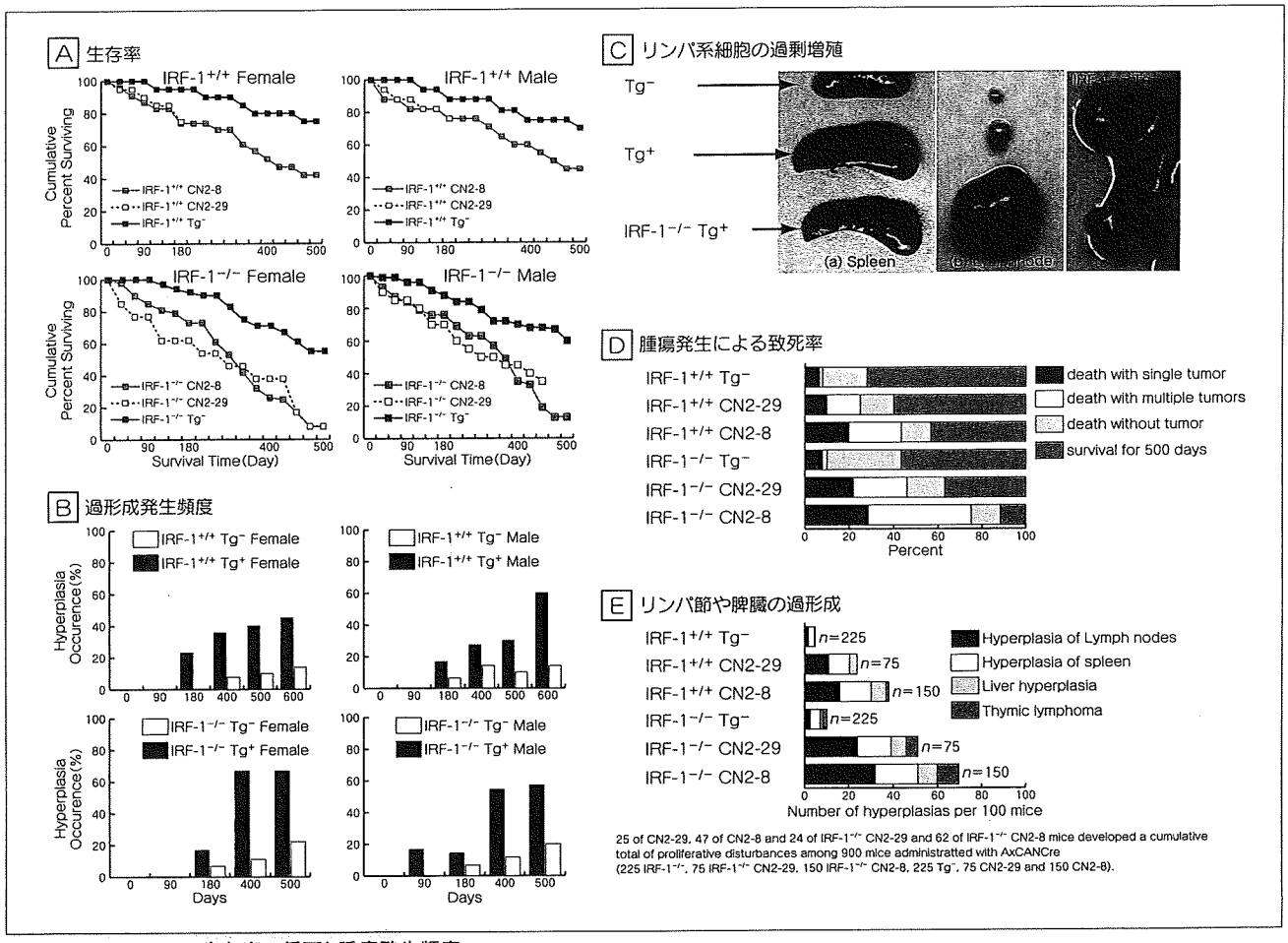

図2　CN2マウスでの生存率の低下と腫瘍発生頻度
A HCV-CN2遺伝子発現マウスは非発現マウスに比較して生存率が低下した.
B 腫瘍発生頻度が顕著に高まった.
C 組織
D 腫瘍による致死率

リンパ性増殖を形成する細胞はB, T細胞の双方で構成されていた. IRF-1欠損マウスではIL-12の産生が低下しており, このうちHCV遺伝子を発現するマウス群では非発現群に比べIL-2やIL-10の産生が顕著に増加していた(図3). さらに, Bcl-2の発現亢進が観察された(図4). HCV遺伝子を発現するリンパ球にIL-2, IL-10を処理するとコロニー形成能が亢進すること, アポトーシス耐性になりカスパーゼ3/7や9の活性が低下すること, Bcl-2の発現が上昇することが明らかとなった. さらにリンパ球でのIL-2やIL-10の産生はコアタンパク質が誘導すること, コアタンパク質存在下でIL-2, IL-10を作用させるとBcl-2の発現が上昇することも明らかとなった.

2. HCVによるBリンパ腫の発生

HCVは肝臓細胞への感染だけではなくリンパ球を始めとする他の臓器に感染する可能性が報告されている[4, 5]. 最近, poly(IC)でHCV遺伝子を発現誘導するpMxCre-CN2マウスを樹立しているが, 本マウスでは肝臓やリンパ球においてHCVを高いレベルで持続発現することが明らかとなった. さらに, 生後180日を過ぎると40%程度のマウスにBリンパ腫が発生することが明らかとなっている[6].

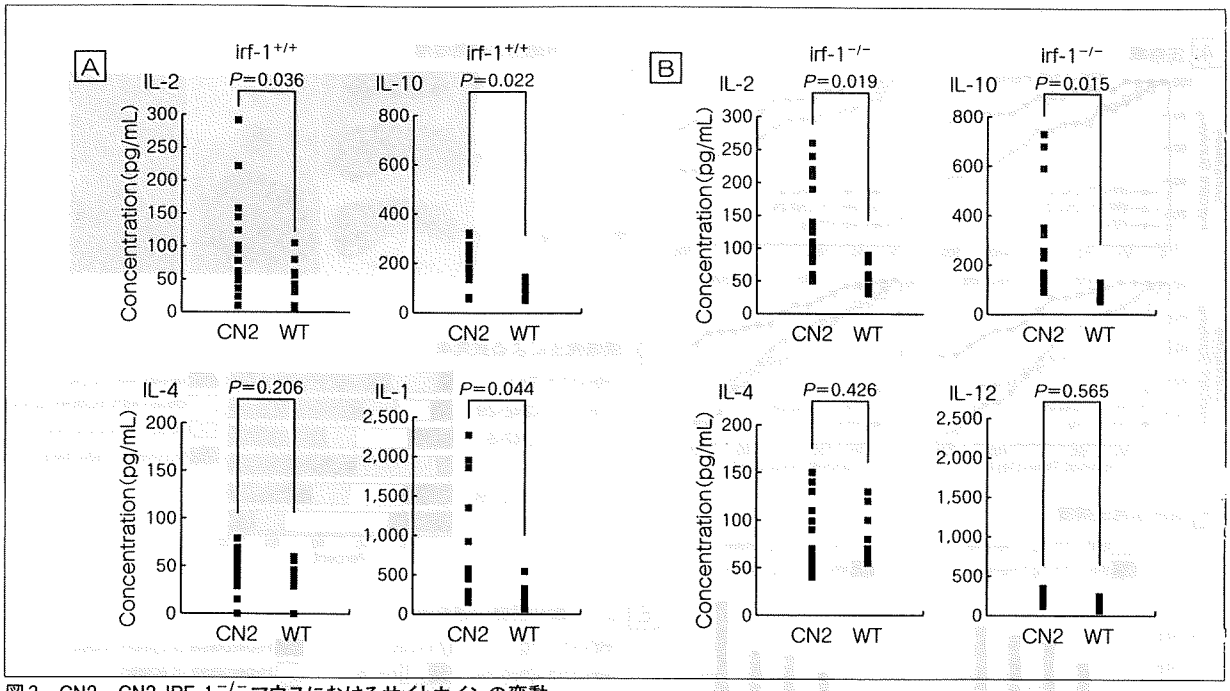

図3 CN2, CN2-IRF-1$^{-/-}$マウスにおけるサイトカインの変動
HCV遺伝子をスイッチング発現し，IRF-1欠損マウスとHCV遺伝子を発現するマウス群で，血清中のIL-2，IL-4，IL-10及びIL-12の量を定量し比較した．

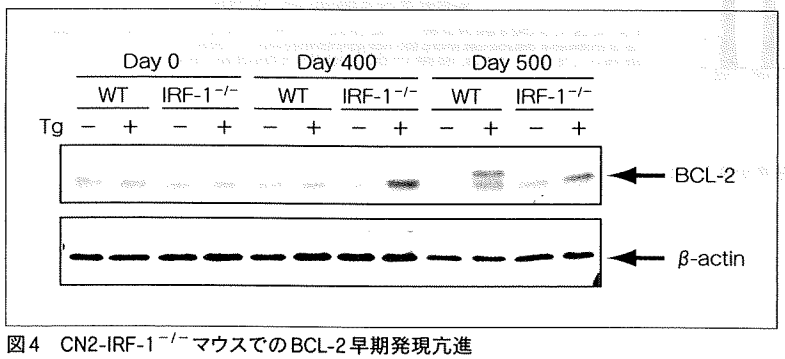

図4 CN2-IRF-1$^{-/-}$マウスでのBCL-2早期発現亢進
リンパ節中のBCL-2をウエスタンブロット法により検出した．

おわりに

CN2 IRF1$^{-/-}$マウス群でのリンパ性増殖発生率が高いことから，IL-2，IL-10のリンパ腫発生における重要性，特にHCV構造蛋白質との共役によるアポトーシス耐性の獲得，Bcl-2蛋白質の発現誘導が明らかとなった．HCV遺伝子がIL-2，IL-10と共役してリンパ性増殖の亢進やアポトーシス抵抗性，コロニー形成能の増加を獲得したのち，セカンドヒットが生じて腫瘍化したリンパ球がリンパ腫を形成するのではないかという可能性を考えてさらに検討を行っている．

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The association between fatal vascular events and risk factors for carotid atherosclerosis in patients on maintenance hemodialysis: Plaque number of dialytic atherosclerosis study

Shinji Maeda[a], Yasunori Sawayama[b], Norihiro Furusyo[a,b], Masaru Shigematsu[c], Jun Hayashi[a,b,*]

[a] Department of Environmental Medicine and Infectious Diseases, Kyushu University, Fukuoka, Japan
[b] Department of General Medicine, Kyushu University Hospital, Fukuoka 812-8582, Japan
[c] Shigematsu Clinic, Fukuoka, Japan

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Vascular motality

ABSTRACT

Atherosclerotic vascular diseases are a major cause of morbidity and mortality for end-stage renal disease patients. We followed prospectively 226 hemodialysis patients by carotid ultrasonography to determine if ultrasonographic markers are predictive of the prognosis of these patients. The end-point was death or completion of the five-year follow-up period. Fatal cerebrovascular and cardiovascular events were the most common cause of death. By multivariate analysis, diabetes mellitus (DM) ($P=0.005$), plaque number (PN) by ultrasonography ($P=0.023$), age ($P=0.001$), calcium–phosphate product ($P=0.049$), and serum albumin ($P=0.009$) were extracted as independent risk factors. The five-year increase of PN was significantly greater for DM patients than for non-DM patients. Moreover, PN was an independent marker of a fatal event, irrespective of DM status. Our results suggest that PN may be a useful predictor of the long-term prognosis of hemodialysis patients.

1. Introduction

Atherosclerotic vascular diseases are a major cause of morbidity and mortality for end-stage renal disease patients [1]. The traditional risk factors for atherosclerotic disease, such as hypertension, dyslipidemia, diabetes mellitus (DM), and smoking, are well known for the general population. Our previous study revealed that the prevalence of carotid atherosclerosis by ultrasonography was significantly higher in hemodialysis patients than in the general population [2]. Other authors have reported non-traditional risk factors such as inflammation [3,4] and the hemodialysis procedure itself [5–8].

Diabetic nephropathy has become increasingly common among patients on maintenance hemodialysis in Japan [1]. The number of patients with DM undergoing hemodialysis was about 41.3% of all patients to who were newly introduced hemodialysis in 2004, and has exceeded the number of patients with chronic renal failure since 1998. The number of patients with diabetic nephropathy on hemodialysis each year is about 14,000 in Japan [1]. DM patients undergoing hemodialysis have more advanced carotid artery lesions than non-DM patients [2].

High-resolution B-mode ultrasonography has made possible the noninvasive evaluation of common carotid artery intima-media thickness (CCA-IMT). CCA-IMT has become widely accepted as a marker of generalized atherosclerosis [9–12] and an association has been made with the occurrence of future vascular events.

The aim of this five-year prospective study was to reconfirm the role of the traditional risk factors for atherosclerosis by ultrasonographic measurement of the number of plaques (plaque number: PN), CCA-IMT, and plaque score (PS) [12] as predictors of long-term risk. We also hoped to clarify the relationship between the putative risk factors and the progression of carotid atherosclerosis in Japanese hemodialysis patients, which is important for more precise assessment of the prognosis.

2. Methods

2.1. Study design

This prospective study was done to evaluate the five-year follow-up of 226 hemodialysis patients. Throughout this study, all

* Corresponding author at: Department of General Internal Medicine, Kyushu University Hospital, Fukuoka 812-8582, Japan. Tel.: +81 92 642 5909; fax: +81 92 642 5916.
E-mail address: hayashij@genmedpr.med.kyushu-u.ac.jp (J. Hayashi).

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hemodialysis patients received the best medical and surgical care available at the time.

2.2. Recruitment and follow-up

The profile of the patients in 2000 is described in our previous report [2]. Each patient was on regular dialysis (4–5 h three times per week) at baseline of the study (duration: 108.4 ± 82.7 months, range: 1–348 months). The dialysate contained 140 mEq/l of sodium, 2.0 mEq/l of potassium, 3.0 mEq/l of calcium, 1.0 mEq/l of magnesium, 110 mEq/l chloride, 30 mEq/l of bicarbonate, and 100 mg/dl of glucose. The lost to follow-up, deceased, and surviving patients are summarized in Fig. 1. The 226 hemodialysis patients (124 male and 102 female; mean age 60.4 ± 13.2 years, range: 22–86 years) were assessed at two dialysis units in Fukuoka Prefecture. Of the 226 hemodialysis patients, 30 were unavailable for follow-up, including 23 who were transferred to other hospitals and 7 who underwent kidney transplantation after the day of examination by carotid ultrasonography. Of the 124 male patients, 28 (22.6%) had DM, 112 (90.3%) had hypertension, 12 (9.6%) had hyperlipidemia, and 50 (40.3%) had a history of a vascular event. Of the 102 female patients, 23 (22.5%) had DM, 83 (81.4%) had hypertension, 12 (11.8%) had hyperlipidemia, and 32 (31.4%) had a history of a vascular event. Hyperlipidemia was seen in 24 (10.6%) patients including 12 (4 male, 8 female) who were untreated, and 12 (8 male, 4 female) who were treated with a statin based antihyperlipidemia drug.

Over the course of the study, 73 of the 226 patients (32.3%) died within the five-year period, with a mean follow-up of 2.29 (range: 0.03–4.98 years) and 167.2 person-years. The 123 surviving patients have a follow-up of five-year period, and 614.9 person-years of follow-up. The 30 lost to follow-up had a mean follow-up of 4.70 (range: 0.50–5.00 years) and 102.8 person-years of follow-up. There were 884.9 person-years of follow-up in this study, and a mean survival period of 3.92 years. All patients were Japanese and informed consent was obtained. The study was done in accordance with the principles of the Declaration of Helsinki.

2.3. End-points

Briefly, the first end-points of the study were a fatal event (vascular event including cerebrovascular and cardiovascular events, infection, malignancy, cardiac failure, or another illness as the main cause of death). Of the 73 who had died, 20 patients (27.4%) died of a vascular event (15 of cerebrovascular and 5 of cardiovascular events) which were defined as the second end point, 15 (20.5%) of infectious disease (8 of pneumonia, 4 of sepsis, 2 of shunt infection, one of cerebral abscess), 8 (11.0%) of malignancy (4 of digestive cancer, 2 of leukemia, one of spine cancer, one of kidney cancer), 7 (9.6%) of sudden death, and 23 (31.5%) of other causes (6 of cardiac failure, 4 of electrolyte abnormality, 2 of ileus, 2 of gastrointestinal bleeding, one of hepatic failure, one of thrombosis, one of acute pancreatitis, 4 patients who changed hospitals for whom the cause of death was unknown, and 2 of accidents).

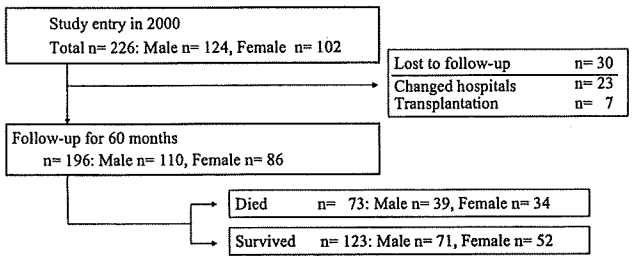

Fig. 1. Flow chart of 226 hemodialysis patients. Thirty patients were unable to be followed. The main causes of death among the 73 patients who suffered a fatal event were vascular events, infection, heart failure, and malignancy.

2.4. Medical history and lifestyle

Data were compiled from medical records and a questionnaire that included personal medical history, family history, and lifestyle habits. Pre-dialysis and post-dialysis blood pressure was measured three times per week at rest for five weeks in 2000, after which the mean blood pressure was calculated for each patient. Hypertension was defined as mean systolic pressure ≥140 mmHg, mean diastolic pressure ≥90 mmHg, or treatment with antihypertensive medications. Hyperlipidemia was defined as either total cholesterol ≥220 mg/dl or receiving lipid-lowering therapy. DM was defined as treatment with anti-diabetic agents or insulin or a past history of DM. Body mass index (BMI) was calculated as the weight in kilograms divided by the height in square meters. A 12-lead electrocardiogram was recorded, and evidence of left ventricular hypertrophy (LVH) was assessed using the Sokolow–Lyon criteria.

2.5. Laboratory parameters

All blood samples were obtained immediately before hemodialysis and stored at −20 °C until analysis. Total cholesterol, triglycerides, and creatinine (enzymatic method), high-density lipoprotein cholesterol (homogeneous assay method), albumin (bromcresol green method), phosphorus (Fiske-Subbarow method), magnesium (xylidyl blue method), intact parathyroid hormone (immunoradiometric assay), Qualitative C-reactive protein (CRP) (turbidimetric immunoassay), and the hemoglobin, hematocrit, and white blood cell count (autoanalyzer) were measured by a commercial laboratory (CRC, Fukuoka, Japan). Qualitative CRP ≥0.5 mg/dl was defined as positive. Low-density lipoprotein cholesterol was calculated according to the Friedewald formula. All assays were done blinded to clinical data and the results of ultrasound examination.

2.6. Carotid ultrasound

Of the 123 surviving patients, 10 declined to undergo carotid ultrasonography in 2005, so data from these patients were only used for comparison of characteristics at baseline and for drawing the survival curve.

Thus, the study included 113 patients who had carotid ultrasonography between 2000 and 2005. Carotid artery lesions were assessed by high resolution B-mode ultrasonography with a 7.5 MHz linear array probe (SSD-1700, Aloka, Tokyo, Japan), as described previously. All examinations of the carotid arteries were done by the leading author, a well trained physician, without any knowledge of patient history or risk factor profile. Each subject was examined in the supine position in a semi-dark room. Both longitudinal and transverse images of the right and left carotid arteries were obtained in the anterior oblique, lateral, and posterior oblique planes. CCA-IMT was defined as the distance between the lumen–intima interface and the media–adventitia interface on B-mode images. Using the probe at an antero-oblique angle, the far wall of the carotid artery was visualized bilaterally in the common carotid artery (CCA-IMT: 20–50 mm proximal to the bifurcation of blood flow), the carotid bulb (0–20 mm proximal to the bifurcation of flow), and the internal and external carotid arteries (ICA and ECA: 0–20 mm distal to the bifurcation).

CCA-IMT was measured at 20, 25, and 30 mm proximal to the bifurcation of flow at the far wall of the right and left common

carotid arteries at end-diastole, and CCA-IMT was calculated as the mean value for each subject. We defined a plaque as a focal CCA-IMT thickening with advanced fibrofatty lesions but significant calcification and/or thrombosis, a scan area with CCA-IMT ≥1.1 mm, and plaque was detected in the internal or external carotid artery-IMT, or CCA-IMT on the right and left side. The PN was calculated by counting the number of plaques in the bilateral carotid arteries in the scanned area. The PS was calculated by totaling the maximal thickness values of all plaques in the scanned area, and categorized as normal (PS < 1.1), mild (PS = 1.1 to <5), moderate (PS = 5 to <10), and severe (PS ≥ 10) [12]. The increase in plaque over the five-years was defined as ΔPN, and percentage change of IMT progression was calculated by the following formula: progression rate = (value at five-year − baseline value) × 100/baseline value.

2.7. Statistical analysis

The survival time for each participant was calculated from the date of initial ultrasonography to the date of death or the end of follow-up for 60 months, whichever came first. The first end-point was defined as all cause death including the second end-point which defined vascular death as cerebro- and cardiovascular death. Two categorical variable comparisons were done by the Fisher's exact test. The mean values of numerical variables were compared by the unpaired *t*-test or the Mann–Whitney *U*-test, the paired *t*-test, or the Wilcoxon-test. The predictors of a fatal event included in multivariate analysis are as follows; age, sex, hypertension, hyperlipidemia, DM, dialysis duration, BMI, PN, CCA-IMT, PS (0/1: normal and mild/moderate and severe), serum albumin, serum total protein, serum qualitative CRP, serum

Table 1
Characteristics.

	Deceased patients (n = 73) mean ± S.D.		Surviving patients (n = 123) mean ± S.D		P-value#	Patients lost to follow-up (n = 30) mean ± S.D	
Physical condition							
Male, n (%)	39	53.4	71	57.7	0.558	14	46.7
Age (years)		67.9 ± 9.0		66.8 ± 12.9	<0.001		56.4 ± 15.1
Smoker, n (%)	34	46.6	52	42.3	0.558	12	40.0
Alcohol cosumption, n (%)	16	21.9	40	32.5	0.112	7	23.3
BMI (kg/m^2)		19.9 ± 2.6		20.2 ± 2.5	0.409		20.7 ± 3.6
Blood pressure (mmHg)							
Systlic		159.4 ± 25.6		150.2 ± 21.5	<0.001		147.1 ± 19.4
Diastolic		78.9 ± 12.6		78.8 ± 11.7	0.950		81.1 ± 8.7
Dialysis duration (month)		103.7 ± 75.9		114.1 ± 89.2	0.387		96.6 ± 70.2
LVH, n (%)	28	38.4	31	25.2	0.052	5	16.7
Present history							
Diabetes mellitus, n (%)	27	37.0	19	15.4	0.001	5	16.7
Hypertension, n (%)	67	91.8	103	83.7	<0.001	25	83.3
Hyperlipidemia, n (%)	4	5.5	12	9.8	0.290	8	26.7
Past history							
Cardiovascular events, n (%)	14	19.2	10	8.1	0.023	5	16.7
Cerebrovascular events, n (%)	24	32.9	23	18.7	0.025	6	20.0
Blood sample data							
Total cholesterol (mg/dl)		156.6 ± 32.8		159.5 ± 30.4	0.535		182.5 ± 35.3
HDL-C (mg/dl)		46.8 ± 15.6		45.8 ± 12.3	0.632		50.3 ± 15.3
LDL-C (mg/dl)		88.5 ± 26.4		90.2 ± 23.9	0.644		106.2 ± 28.0
Triglycerides (mg/dl)		106.2 ± 63.4		117.0 ± 59.1	0.232		130.0 ± 80.2
Total protein (g/dl)		7.0 ± 0.5		7.0 ± 0.5	0.932		7.1 ± 0.4
Albumin (g/dl)		3.8 ± 0.3		3.9 ± 0.3	<0.001		3.9 ± 0.3
Creatinine (mg/dl)		8.91 ± 2.12		11.06 ± 2.14	<0.001		10.69 ± 2.35
Uric acid (mg/dl)		8.8 ± 9.2		8.1 ± 1.3	0.376		8.2 ± 1.4
Calcium (mg/dl)		9.5 ± 0.9		9.5 ± 0.9	0.961		9.8 ± 1.3
Phosphorus (mg/dl)		5.6 ± 1.5		5.6 ± 1.3	0.997		5.3 ± 1.4
Magnesium (mg/dl)		2.7 ± 0.5		2.7 ± 0.4	0.482		2.9 ± 0.4
Calcium–phosphate product (mg/dl)^2		54.3 ± 16.2		53.3 ± 14.7	0.666		51.8 ± 14.8
Intact parathyroid hormone (pg/dl)		178.7 ± 212.2		166.8 ± 180.4	0.677		152.2 ± 157.8
Hemoglobin (g/dl)		9.8 ± 1.2		9.9 ± 1.1	0.662		10.0 ± 0.9
Hematocrit (%)		30.7 ± 3.4		30.9 ± 3.1	0.630		31.1 ± 2.8
White blood cell (×10^3/1)		5788 ± 1678		5818 ± 1687	0.903		5967 ± 1269
CRP (>0.5 mg/dl), n (%)	9	12.3	10	8.1	0.337	4	13.3
Ultrasonographic findings							
Prevalence of plaque, n (%)	72	98.6	103	83.7	0.001	24	80.0
Calcification plaque, n (%)	69	94.5	87	70.7	<0.001	23	76.7
PN		4.63 ± 3.18		2.54 ± 2.07	<0.001		2.97 ± 2.48
CCA-IMT(mm)		0.97 ± 0.16		0.86 ± 0.16	<0.001		0.87 ± 0.16
PS (nomal, mild/moderate, severe)		23/50		75/48			18/12
PS (moderate, severe; %)	50	68.5	48	39.0	<0.001	12	40.0

Values presented as mean value ± S.D. and number followed by (%).
Patients lost to follow-up were 23 who changed hospitals and 7 who received kidney transplantation.
BMI: body mass index, LVH: left ventricular hypertrophy, HDL-C: High density lipoprotein-cholesterol.
LDL-C: Low density lipoprotein-cholesterol, CRP: C-reactive protein.
CCA-IMT: common carotid artery intima-media thickness, PN: plaque number,
PS: plaque score; PS < 1.1 mm: normal, 1.1 ≤ PS < 5.0 mm: mild, 5 ≤ PS < 10 mm: moderate, PS ≥ 10.0 mm: severe.
Comparing deceased patients and surviving patients.

calcium–phosphate product, serum intact parathyroid hormone, serum uric acid, history of cerebrovascular and cardiovascular disease, prevalence of LVH. Mortality was compared by Kaplan–Meier analysis and log rank statistics, with 95% confidence intervals (95% CI). The explanatory variables for DM, PN, age, calcium–phosphate product, and albumin were extracted using the Cox regression analysis. The relative risks and their 95% CI were calculated using the estimated regression coefficients and standard errors. In addition, time-to-fatal event curves were compared by the log–rank test and were used to estimate the absolute risk over five years for each of the ultrasound values. Adjustment variables were chosen for the multiple regression model based on the possibility that the covariate of interest may be associated with the risk of mortality. All statistical calculations were done with the Biomedical Computer Programs-P (BMDP) Statistical Software (Release 7.1, SUN/UNIX). A probability (P) value <0.05 was considered statistically significant.

3. Results

3.1. The characteristics of 73 deceased, 123 surviving, and 30 patients lost to follow-up (Table 1)

The prevalence of a history of DM (37.0%), hypertension (91.8%), and past history of a cardiovascular (19.2%), or cerebrovascular event (32.9%) at baseline were significantly higher in the deceased than in surviving patients (15.4%, 83.7%, 8.1%, and 18.7%, respectively). Additionally, the mean systolic blood pressure (159.4 mmHg) at baseline was significantly higher in the deceased than in the surviving patients (150.2 mmHg). However, the mean serum albumin (3.8 mg/dl) and the mean creatinine (8.91 mg/dl) were significantly lower in the deceased than in the surviving patients (3.9 mg/dl and 11.06 mg/dl, respectively). In carotid ultrasonographic findings, the mean PN (4.63), the mean CCA-IMT (0.97 mm), the prevalence of moderate and severe groups of PS (68.5%), plaque (98.6%), and calcification (94.5%) were significantly higher in the deceased than in the surviving patients (2.54, 0.86 mm, 39.0%, 83.7%, and 70.7%, respectively). There were no significant differences between the deceased patients in relation to smoking, alcohol consumption, sex, BMI, LVH, duration of hemodialysis, serum total cholesterol, high density lipoprotein, triglycerides, uric acid, intact PTH, qualitative CRP, mineral, or hematological values.

3.2. Multivariate analysis for the development of a fatal event

The Cox regression analysis with backward stepwise regression of 226 hemodialysis patients showed the significantly independent risk factors at baseline for all cause death to be a history of DM (P=0.005), PN (P=0.023), age (P=0.001), calcium–phosphate product (P=0.049), and serum albumin (P=0.009) (Table 2).

The five-year survival rate was compared for four groups, classified by the PN by ultrasonography (0–1, 2, 3–4, and ≥5) and by DM status, and was adjusted for independent factors such as age, serum calcium–phosphate product, albumin, and related covariates. The highest group of PN ≥5 had a significantly lower survival rate than the other groups (log–rank test, P<0.001) (Fig. 2). The survival rate of DM patients was significantly lower than that of non-DM patients (log–rank test, P<0.001) (Fig. 3). To clarify the effect of PN and DM on survival, the combined risk factors for PN and DM were divided into four groups: PN <5 and non-DM (group A, n=141), PN <5 and DM (group B, n=33), PN ≥5 and non-DM (group C, n=34), PN ≥5 and DM (group D, n=18). The five-year survival rate by all cause mortality (Fig. 4a) and the five-year survival rate by vascular mortality (Fig. 4b) were adjusted for independent markers such as age, dialysis duration, albumin, and uric acid (Table 3). The five-year survival rate of group A was higher than that of the other groups (all $P \leq 0.001$). Moreover, the all cause and vascular mortality of group

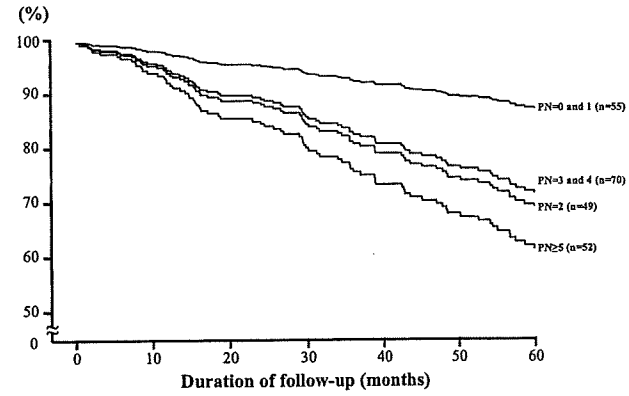

Fig. 2. The five-year survival rates of hemodialysis patients grouped by plaque number (PN). The five-year survival rate of patients with PN ≥5 was significantly lower than that of the other three patient groups (log–rank test: P<0.001).

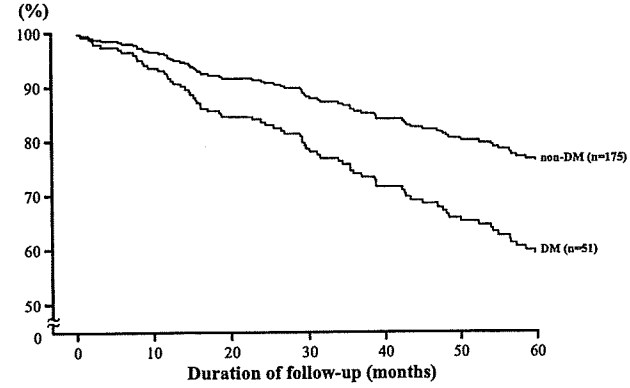

Fig. 3. The five-year survival rates of hemodialysis patients with and without diabetes mellitus (DM). The five-year survival rates of patients with DM (n=51) was a significantly lower than that of non-DM patients (n=175) (log–rank test: P<0.001).

Table 2
Cox regression analysis with backward stepwise regression of the risk factors for a fatal event in 226 hemodialysis patients.

Parameters		β	Relative risk	95% CI		P-value
				Lower	Upper	
Diabetes mellitus	Yes	0.707	2.029	1.242	3.313	0.005
Plaque number	1	0.100	1.105	1.014	1.205	0.023
Age	1 y.o.	0.041	1.042	1.017	1.068	0.001
Ca × P	1 (mg/dl)^2	0.014	1.014	1.000	1.029	0.049
Abumin	1 g/dl	−1.165	0.312	0.131	0.745	0.009

95% CI: 95% confidence intervals.
Ca × P: serum calcium–phosphate product.

Table 3
Cox regression analysis with backward stepwise regression for all cause and vascular mortality according to significant risk markers and the combined plaque number (PN) and diabetes mellitus (DM) status.

Parameters		All cause mortality					Vascular mortality				
		β	Relative risk	95% CI		P-value	β	Relative risk	95% CI		P-value
				Lower	Upper				Lower	Upper	
Age	1 y.o.	0.048	1.05	1.02	1.08	<0.001					
Dialysis duration	1 month	0.004	1.00	1.00	1.01	0.029					
Albumin	1 g/dl	−0.978	0.38	0.16	0.89	0.025					
Uric acid	1 mg/dl	0.030	1.03	1.00	1.06	0.031					
group A vs. group B		1.280	3.60	1.78	7.28	<0.001	2.069	7.92	2.23	28.14	0.001
group A vs. group C		0.941	2.56	1.31	5.03	0.006	2.212	9.13	2.57	32.52	0.001
group A vs. group D		1.183	3.27	1.52	7.03	0.003	2.242	9.42	2.35	37.74	0.002

95% CI: 95% confidence intervals. Group A ($n=141$), PN<5 and Non-DM; group B ($n=33$), PN <5 and DM; group C ($n=34$), PN >5 and Non-DM; group D ($n=18$), PN >5 and DM.

C patients were not significantly different than those of group B and D patients (vs. group B, $P=0.336$ and $P=0.802$, respectively; vs. group D, $P=0.783$ and $P=0.944$, respectively). Therefore, PN was an important marker for the survival of hemodialysis patients, irrespective of DM status.

Table 3 shows significant risk parameters of the combined PN and DM for all cause and vascular mortality by Cox regression analysis with backward stepwise regression. The relative risk (RR) of significant parameters for all cause mortality were age (RR: 1.05), dialysis duration (RR: 1.00), serum albumin (RR: 0.38), serum uric acid (RR: 1.03), and group A vs. group B (RR: 3.60), group C (RR: 2.56), group D (RR: 3.27). The RR of significant parameters for vascular mortality were group A vs. group B (RR: 7.92), group C (RR: 9.13), and group D (RR: 9.42). Therefore, these findings suggest that PN ≥5 and DM were related to all cause and vascular mortality.

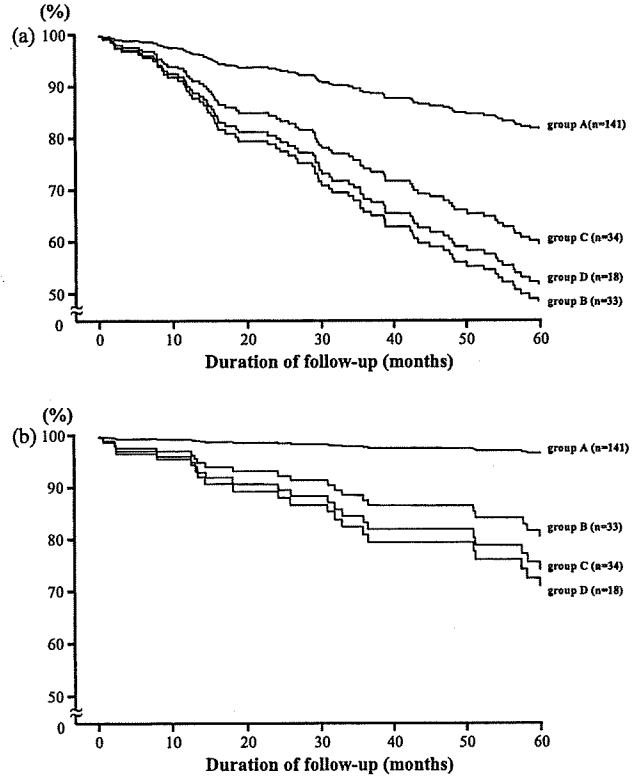

Fig. 4. The survival rate after adjustment for all cause mortality (a) and for vascular mortality (b) adjusted for age, calcium–phosphate product, and albumin. The five-year survival rates of hemodialysis patients grouped by plaque number (PN) and diabetes mellitus (DM) status (group A: $n=141$, DM and PN <5; group B: $n=33$, DM and PN ≥5; group C: $n=34$, non-DM and PN <5; group D: $n=18$, non-DM and PN ≥5). The survival rate after adjustment for all cause and vascular death in group A was significantly higher than that of the other three groups (log–rank test: both $P\leq 0.001$). Moreover, the all cause and vascular mortality of group C patients were not significantly different than those of group B and D patients (vs. group B, $P=0.336$ and $P=0.802$, respectively; vs. group D, $P=0.783$ and $P=0.944$, respectively).

3.3. *Change of carotid atherosclerosis from baseline to follow-up for the 113 surviving patients (Table 4)*

The mean PN and CCA-IMT levels were adjusted for independent factors such as age, calcium–phosphate product, and albumin by Cox regression analysis with backward stepwise regression. The mean PN in 2005 (at follow-up) of both DM and non-DM patients were significantly higher than those in 2000 (at baseline) (both $P\leq 0.001$). Notably, the mean PN of DM patients was significantly higher than that of non-DM patients at both baseline and follow-up ($P=0.019$ and $P\leq 0.001$, respectively). Moreover, the increase of PN (ΔPN) during the follow-up period was significantly higher (4.81 ± 0.55) in DM patients than in non-DM patients (2.83 ± 0.23) ($P\leq 0.001$).

In the case of DM patients, the mean CCA-IMT was also significantly higher (1.14 ± 0.05 mm) at follow-up than at baseline (0.94 ± 0.03 mm) ($P=0.017$). The mean CCA-IMT of DM patients was significantly higher than that of non-DM patients at both baseline and follow-up ($P=0.010$ and $P\leq 0.001$, respectively), and the mean progression rate of CCA-IMT of DM patients was significantly greater ($22.4\pm4.9\%$) than that of non-DM patients ($1.7\pm2.0\%$) ($P\leq 0.001$). However, there was no statistically significant increase of the mean CCA-IMT of the 96 non-DM patients between baseline and follow-up (0.84 ± 0.01 mm and 0.82 ± 0.02 mm, $P=0.090$). DM patients had significantly higher frequency of PS severity of moderate/severe than that non-DM at baseline ($P=0.00$). Additionally, all 5 DM patients who had normal or mild PS at baseline changed to moderate or severe PS at follow-up, and 37 of 67 (55.2%) of non-DM patients who had normal or mild PS changed to moderate or severe PS.

4. Discussion

The present study of three ultrasonographic parameters, PN, CCA-IMT, and PS, revealed new information that may be useful for the evaluation of carotid atherosclerotic risk factors related to a fatal event, including all cause and vascular death, and the prognosis of Japanese hemodialysis patients. We found that the follow-up PN was significantly increased in comparison with the baseline PN, but that the CCA-IMT of non-DM patients was unchanged, CCA-IMT and PS were not independent risk markers in a multivariate analysis. We also found the predictive risk markers for all cause and vascular death by ultrasonography to be associated with PN parameters,

Table 4
Changes of carotid atherosclerosis.

	DM (n=17)		non-DM (n=96)		p - value
PN (mean ± S D)					
2000	3.46 ± 0.45	< 0.001[a]	2.33 ± 0.18	< 0.001[b]	0.019[c]
2005	8.18 ± 0.61		5.17 ± 0.25		< 0.001[d]
ΔPN	4.81 ± 0.55		2.83 ± 0.23		< 0.001[e]
CCA-IMT (mm, mean ± S D)					
2000	0.94 ± 0.03	0.017[a]	0.84 ± 0.01	0.090[b]	0.010[c]
2005	1.14 ± 0.05		0.82 ± 0.02		< 0.001[d]
Progression rate (%)	22.4 ± 4.9		1.7 ± 2.0		< 0.001[e]

PN: plaque number, CCA-IMT: common carotid artery-intima-median thickness. The increase in plaque over the five-years was defined as ΔPN. percentage change of IMT progression was calculated by the following formula: progression rate = (value at five-year − baseline value) × 100/baseline value. [a]Comparing atherosclerotic change of DM patients in 2000 and 2005. [b]Comparing atherosclerotic change of non-DM patients in 2000 and 2005. [c]Comparing DM and non-DM in 2000. [d]Comparing DM and non-DM in 2005. [e]Comparing five-year changes of DM and non-DM.

irrespective of DM status. Therefore, PN was an important marker for the survival of hemodialysis patients.

Benedetto et al. [16] reported that mean-IMT was an independent predictor of cardiovascular death among end-stage renal failure patients. However, our study documented no relation between CCA-IMT and mortality. This is because we more strictly defined vascular death as stroke and myocardial infarction excluding sudden death, heart failure, embolism, and aortic aneurysm. Although we used a 6 point average of CCA-IMT as in Benedetto et al., the following two points differed between our study and theirs. Firstly, the percentage of the studied patients with DM in our study (22.6%) was higher than in Benedetto et al. (6.5%). Secondly, the methodology of our study was classified according to DM status, and Benedetto's study included DM status and both hemodialysis and continuous ambulatory peritoneal dialysis. Therefore, we believe our study adds useful data for comparison with that of the previous report.

Some studies have shown that the rate of vascular events of hemodialysis patients is substantially higher than that of the general population (2.4–18.0%) [11,21], moreover, hemodialysis patients are characterized by an exceptionally high mortality rate by such diseases [7–12]. Of 226 hemodialysis patients, 73 (32.3%) in this study died within the five-year period, and 20 of the deceased patients (27.4%) had had a fatal vascular event, similar to that of studies from other laboratories, which found rates of 16–42% [7,18].

Complication with DM is a reproducible, traditional risk factor associated with the progress of atherosclerosis [9,18–19,22]. Some previous studies have shown that DM and/or hypertension are significantly involved in such fatal events [5,9,10,21], and that patients with both DM and ESRD have significantly higher CCA-IMT values than those with either DM or ERSD alone [9]. DM was also found to be strongly associated with a fatal event in this study. Many previous studies have used ultrasonography to assess risk factors for the progression of carotid atherosclerosis in the general population [2,13–15] and hemodialysis patients [2,5,7–12,16,17].

Our study demonstrated that PN was clearly correlated with all cause mortality. Moreover, PN was strongly associated with vascular mortality, irrespective of DM status, and the relative risk of non-DM patients with PN ≥5 was the same risk of DM for all cause and cardiovascular death, by multivariate analysis. These results suggest that PN is one of the most important markers for the prognosis of hemodialysis patients. None of the other ultrasonographic parameters were predictive in this study.

The mechanisms for the accelerated atherosclerosis of hemodialysis patients may be inflammation associated with dialysis-related causes [3,4,6,20,23] and/or the hemodialysis procedure itself [5–8]. One study reported that the dialysis procedure causes progressive endothelial cell injury that leads to both macro- and micro-vascular disease [13]. However, the reason for the increase of the PN in hemodialysis patients is not clear.

There are several limitations to this study. First, we were not able to classify the cause of death of 11 (15.1%) of the 73 deceased patients (7 sudden deaths and 4 for whom cause of death was unknown because of a change of hospitals), and we could not do ultrasonographic follow-up of 10 of the 123 surviving patients (8.1%) in 2005. Second, there were only two dialysis units, so the results would have only internal validity. A future, large-scale study with long-term observation will be necessary to fully clarify the risk factors of the fatal events of hemodialysis patients.

The present study revealed that PN by ultrasonography was an important marker for the survival of hemodialysis patients.

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Original Article

Association of *Chlamydophila pneumoniae* DNA in Peripheral Blood Mononuclear Cells and IgA Antibody with Atherosclerotic Diseases

Yasunori SAWAYAMA[1], Kensuke KIKUCHI[2], Masafumi TATSUKAWA[2], Shin HAYASHI[2], Yuuji TAIRA[3], Norihiro FURUSYO[1] and Jun HAYASHI[1]

[1]*Department of General Medicine, Kyushu University, Fukuoka, Japan*
[2]*Department of General Medicine, Harasanshin hospital, Fukuoka, Japan*
[3]*Department of Cardiovascular Medicine, Harasanshin hospital, Fukuoka, Japan*

Abstract An association has been demonstrated between *Chlamydophila pneumoniae* (*C. pneumoniae*) infection and atherosclerosis, but data on the relationship between *C. pneumoniae* DNA in peripheral blood mononuclear cells (PBMC) and antibodies to this organism are lacking. We investigated the *C. pneumoniae* DNA in PBMC by polymerase chain reaction (PCR) and *C. pneumoniae* IgG and IgA antibodies by enzyme-linked immunosorbent assay of 168 patients with atherosclerotic diseases and 27 controls (healthy control subjects). *C. pneumoniae* DNA was detected for 48/168 (29%) atherosclerosis patients, IgG for 79 (47%), and IgA for 98 (58%), whereas the corresponding numbers for the controls were 11 (41%), 13 (48%), and 7 (26%). There was no significant difference of the *C. pneumoniae* DNA positivity rate between the atherosclerosis patients and the controls. However, the *C. pneumoniae* IgA-positive rate was significantly higher for carotid atherosclerosis patients who had *C. pneumoniae* DNA in their PBMC than for those without it (74% vs. 18%, $P < 0.05$). Among the patients with coronary artery disease, the *C. pneumoniae* IgA antibody positive rate was significantly higher for the patients with DNA than for those without it (68% vs. 18%, $P < 0.05$). Our results suggest that a high *C. pneumoniae* IgA antibody titer and *C. pneumoniae* DNA positivity are associated with an increased risk of atherosclerotic diseases due to endovascular *C. pneumoniae* infection.

Key words : *Chlamydophila pneumoniae* ; Atherosclerosis ; Peripheral blood mononuclear cells ; Polymerase chain reaction ; Antibody

Introduction

Chlamydophila pneumoniae (*C. pneumoniae*), an obligatory intracellular pathogen, is a common cause of respiratory tract infection[1)2)]. Several studies have already shown the presence of *C. pneumoniae* in blood stream of healthy volunteers and have indicated that more than half of the adult population has been exposed to this organism, with infection and reinfection occurring during their lifetime[1)~3)].

Atherosclerosis is a highly prevalent disease, and it is currently the greatest cause of morbidity and mortality in developed societies. Many risk factors have long been identified as contributing to the development of atherosclerosis that manifests as coronary artery disease (CAD) and myocardial infarction (MI). More recently, the possibility has been raised that infectious agents may trigger a cascade of biological and biochemical reactions leading to inflammation, atherogenesis, and vascular thrombosis. A serological association between *C. pneumoniae* and CAD was

Address for Correspondence : Jun HAYASHI, M.D., Ph.D.
Department of General Medicine, Kyushu University Hospital, Higashi-ku, Fukuoka 812-8582, Japan
TEL : + 81-92-642-5909
FAX : + 81-92-642-5916
E-mail address : hayashij@genmedpr.med.kyushu-u.ac.jp

first demonstrated by Saikku *et al.* in 1988[4]. This association has been confirmed by subsequent studies[5], although several authors[6)~8)] have failed to find any association. We[9] previously studied a relationship between *C. pneumoniae* infection and the effect of lipid-lowering drugs on the carotid atherosclerosis (CA) of hypercholesterolemic patients.

C. pneumoniae infection reduced the effect of lipid-lowering therapy on CA, indicating that this organism may play a role in the progression of atherosclerosis. Moreover, *C. pneumoniae* has been detected in atherosclerotic tissues by polymerase chain reaction (PCR), immunohistochemistry, electron microscopy, culture, and other techniques[10].

C. pneumoniae infection generally starts in the respiratory tract, and the organisms within alveolar macrophages are probably spread systemically through the bloodstream[11)12)]. The elementary body, the metabolically inactive extracellular stage of the life cycle of chlamydiae, has never been found circulating freely in the blood, but monocytes/macrophages may carry *C. pneumoniae* from the lungs to the arterial walls. In vitro studies have indicated that *C. pneumoniae* can infect and reproduce within human endothelial cells, smooth muscle cells, and macrophages, which are key cell types involved in the process of atherosclerosis[13)14)]. Also, recent studies have detected *C. pneumoniae* DNA in the peripheral blood mononuclear cells (PBMC) of patients with CAD[15]. Because serology alone cannot diagnose vascular infection, direct detection methods based on examination of peripheral blood components may be more useful as markers of infection.

The aim of this study is to evaluate the association between *C. pneumoniae* and atherosclerosis to investigate the prevalence of *C. pneumoniae* DNA within PBMC and *C. pneumoniae* antibodies from patients with various atherosclerotic diseases.

Methods

Subjects

Between May 2001 and July 2002, 168 patients with various atherosclerotic diseases (109 men and 59 women, mean age 67 ± 9 years) and 27 controls (6 men and 21 women, mean age 59.0 ± 7.1 years) were enrolled at Kyushu University Hospital.

Selection of Subjects

All of the patients with atherosclerotic diseases admitted to Kyushu University Hospital (Fukuoka, Japan) were considered eligible for the present study. The type of atherosclerotic disease was CA for 68 patients, stable angina (SA) for 29, acute coronary syndrome (ACS) including acute myocardial infarction and unstable angina for 39, old myocardial infarction (OMI) for 32. The patients with CA had no history of CAD, but they all had an abnormal carotid intima-media thickness (IMT) and/or plaque on ultrasonography (defined as a generalized or focal IMT $\geq$ 1.1 mm, respectively)[8]. Patients with ACS had ischemic chest pain and typical changes on their electrocardiogram (ECG) and/or increased cardiac enzyme levels. Angina patients without clinical evidence of ischemia within the previous one month were defined as having SA. All of the patients with SA, ACS, or OMI (n = 100) underwent coronary angiography.

Exclusion criteria were acute infection, exacerbation of chronic infectious or inflammatory diseases, and severe liver or renal disease.

Informed consent for collection of blood or tissues was obtained from the patients (or their closest relatives). Information on each participant was compiled from the medical records and from a questionnaire about the personal medical history and lifestyle. The design of this study was approved by the Ethics Committee and the Data Protection Committee of Kyushu University Hospital (Fukuoka, Japan).