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Figure Legends

Fig. 1. Study Protocol. All rats are unilaterally nephrectomized on day -1 and divided into 4 groups on day 0.
N group; no injection of reagents. HV group; injection of 3.5mg/kg of Habu snake venom (HV). A group; continuous administration of 100ng/min of Angiotensin II (AII). H+A group; administration of HV and AII.

Fig. 2. Glomerulonephritis is developed with the combination of HV and AII, and HIF-1α is induced in the intact glomeruli.
There are no glomerular or tubular injuries in N group (A), HV group (B), A group (C) and H+A group on day 1 (D). Damaged glomeruli, characterized by extensive mesangiolysis, are observed in H+A group on day 2. PAS staining. Magnification, *100 (E). Focal and segmental mesangiolysis with large capillary aneurysmal ballooning are observed in the H+A group on day 2. PAM staining. Magnification, *400 (F). The number of GN was significantly less in pre-treatment with $CoCl_2$ than without. PAS staining. Magnification, *100 (G). Immunoreactive HIF-1α positive signals are not detected in the N group (H). Nuclear HIF-1α signals are observed in a glomerulus and tubules in the A group. Magnification, *200 (I). A glomerulus in the H+A group on day 2 possesses intact cells with HIF-1α positive signals, in contrast, other parts have few HIF-1α signals due to mesangiolysis. Magnification, *200 (J).

Fig. 3. Semiquantitative analysis of morphologic changes in our glomerulonephritis model. The main lesion in the H+A group is initially detected on day 2 as mesangiolysis in glomeruli; however, there are no tubular lesions of necrosis except for tubular casts; in contrast, there are no morphological changes in the N and A groups. MES, Mesangiolysis score.

Fig. 4. Serum UN, Cr and SBP are increased with the combination of HV and AII. The serum UN (A) and Cr (B) levels in the H+A group on day 2 are significantly higher than other groups. SBP increases significantly with administration of AII (A and H+A groups) (C).

Fig. 5. The protein level of HIF-1α is increased by administration of HV and AII, and pretreatment of $CoCl_2$ increases HIF-1α expression before development of GN. HIF-1α is not detected in the N and HV groups (Day2). However, HIF-1α is detected in A (Day2) and H+A (Days 1 and 2) groups (A). The $CoCl_2$ group, in accord with the level of HIF-1α induction, was divided into two groups. HIF-1α is greatly induced before the development of GN ($CoCl_2$ group Pre-1), and is followed by a high level ($CoCl_2$ group Day2-1); in contrast, it is not efficiently induced ($CoCl_2$ group Pre-2), and also is scarcely detected on day 2 ($CoCl_2$ group Day2-2) (B). The rate of pre-induction of HIF-1α by $CoCl_2$ is comparable with that of the inhibition of GN by $CoCl_2$ (C).

Fig. 6. Pretreatment with $CoCl_2$ attenuates GN. Serum UN (A) and Cr (B) levels in the $CoCl_2$ group on day 2 are significantly decreased compared to those in the non-$CoCl_2$ group. There is no significant difference in SBP between the $CoCl_2$ and non-$CoCl_2$ groups (C).

Fig. 1

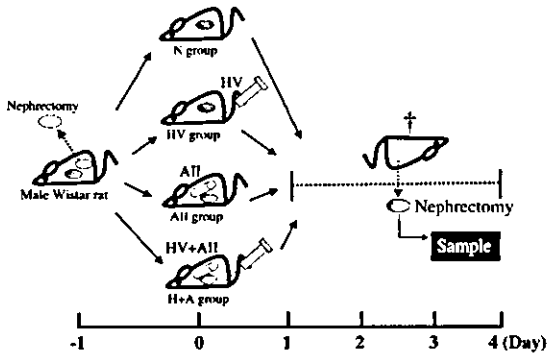


Fig. 2

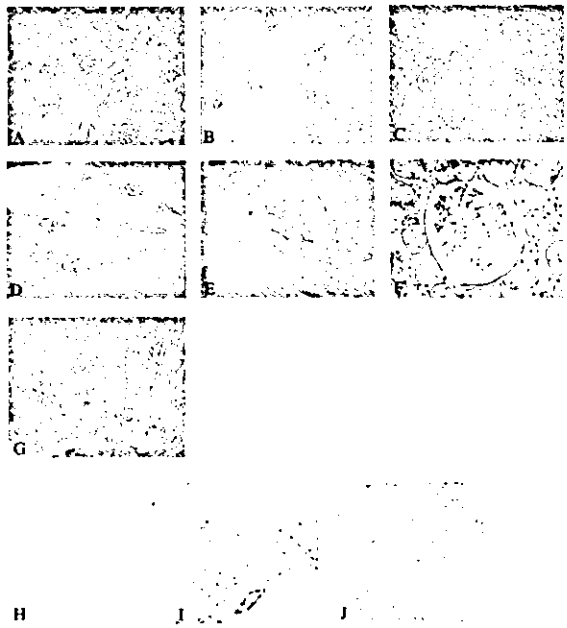


Fig. 3

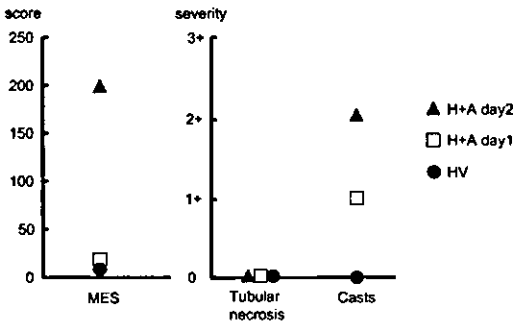


Fig. 3

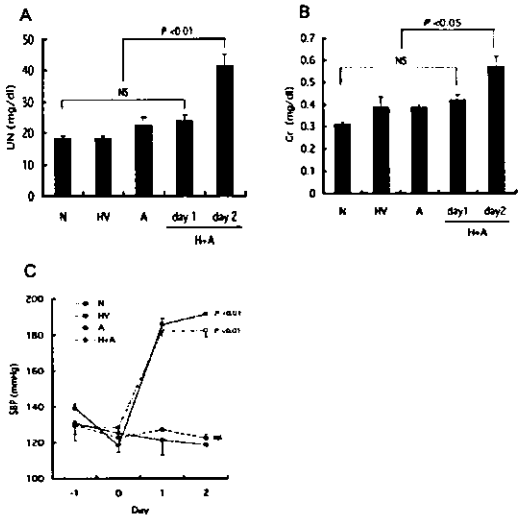


Fig. 4

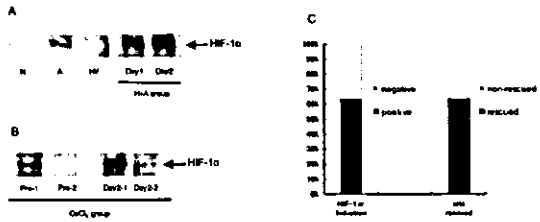
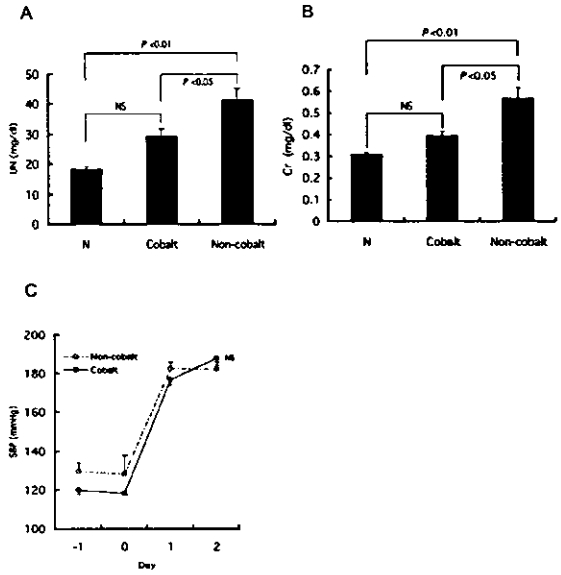


Fig. 5



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ORIGINAL ARTICLE

Association between arterial stiffness and platelet activation

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Increased arterial stiffness is strongly associated with atherosclerosis, while platelet activation is an important trigger of thrombotic events in patients with atherosclerosis. However, little is known about the effect of arterial stiffness on platelet activation. We therefore investigated the association between arterial stiffness and platelet activation in 38 normal volunteers (20 men and 18 women) aged 23–77 years (mean = 49 ± 15 years). Arterial stiffness was assessed by measuring brachial–ankle pulse wave velocity (ba-PWV) and heart–brachial PWV (hb-PWV). Flow cytometric analyses were performed to evaluate platelet activation by measuring surface expression of P-selectin and platelet–neutrophil complexes (PNC) before and after activation by ADP. We also calculated the difference between basal and stimulated states of P-selectin and PNC to assess platelet activation reserve. PWVs were significantly correlated with age and BP ($r = 0.60$–0.81). For platelet activation and activation reserve, correlations with age were less strong but remained significant ($r = 0.36$–0.61), with the exception of P-selectin (not significant, NS), and correlations with SBP were similar ($r = 0.35$–0.53). A significant correlation was found between PWVs and platelet activation ($r = 0.43$–0.74). Multiple regression analysis demonstrated significant correlations between platelet activation and reserve and PWVs (coefficient = 2.17–6.59), when both age and BP were adjusted for simultaneously. In conclusion, platelet activation was associated with arterial stiffness, suggesting that arterial stiffness may play an important role in thrombotic events.
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Keywords: arterial stiffness; pulse wave velocity; P-selectin; platelet–neutrophil complexes

Introduction

Platelet activation and aggregation are important triggers of thrombotic events in patients with atherosclerosis. In such patients, platelets are activated at the site of atheroma due to increased shear stress in the narrowed vessels.[2,3] Increased platelet activation is observed in patients with coronary risk factors and cardiovascular events.[4–12]

Increased arterial stiffness, measured with pulse wave velocity (PWV), has been shown to be associated with atherosclerosis and risk factors of atherosclerotic cardiovascular disease,[13–21] and is an independent predictor of cardiovascular events.[22,23] Therefore, although platelets are likely to be activated in patients with atherosclerotic disease who exhibit increased arterial stiffness, little is known about the relation of arterial stiffness itself to platelet activation.

Recently, platelet activation has been widely evaluated by measuring soluble P-selectin; a platelet surface molecule also termed CD62P.[4,6–8,11] Although the measurement of soluble P-selectin is simple and useful, it is an indirect method of evaluating platelet activation. On the other hand, platelet activation can be detected directly by measuring surface antigen CD62P using flow cytometry.[2,3,5,9,10,12] Furthermore, detection of platelet–neutrophil complexes (PNC), which are formed as a result of interaction with CD62P provides an additional means to detect platelet activation.[24]

The purpose of this study was to investigate the association between arterial stiffness and platelet activation by measuring PWV, P-selectin, and PNC in subjects without atherosclerotic disease.

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Materials and methods

Subjects

We studied 38 healthy nonsmoking volunteers (20 men and 18 women), aged 23–77 years (mean = 49 ± 15 years) with no evidence of heart disease on physical examination, standard 12-lead electrocardiography, chest radiography, echocardiography, or blood chemistry analysis. Subjects had no self-reported past history or current evidence of cardiovascular disease, hypertension, hypercholesterolaemia, diabetes mellitus or renal disease. Basic characteristics of subjects are shown in Table 1. None of the subjects had frequent ectopic beats or atrial fibrillation and none had taken any medication for at least 10 days. Informed consent was obtained before performing the study and the study protocol was approved by the Local Ethics Committee of Kochi Medical School.

Evaluation of arterial stiffness

Arterial stiffness was evaluated by PWV, measured using volume-plethysmographic apparatus (Colin, Komaki, Japan).[18–21] Data were acquired with subjects lying supine in a quiet and temperature-controlled room at 11 AM, at least 3 h after breakfast. Surface electrodes were attached to both wrists for ECG measurement, a microphone was positioned at the left sternal edge to detect heart sounds, and cuffs incorporating plethysmographic and oscillometric sensors were fastened around both the brachial regions and ankles to measure pulse wave forms and blood pressure. Brachial–ankle PWV (ba-PWV) and heart–brachial PWV (hb-PWV) were measured as follows. The time interval between the wave foot of the brachial waveform and that of the ankle waveform was defined as the time interval between the brachial region and ankle, while the time interval between the heart and the right brachial artery was defined as the time interval between the second heart sound and the right brachial waveform. The distance between these sampling points was calculated automatically according to the height of the subject. PWVs were calculated by dividing each distance by the respective time interval. Right brachial blood pressure (systolic and diastolic) and pulse rate were concurrently measured.

Table 1 Clinical characteristics of subjects

Parameters	*All subjects (n = 38)*
Age (years)	49 ± 15
Gender, male/female	20/18
Systolic blood pressure (mmHg)	125 ± 16
Diastolic blood pressure (mmHg)	77 ± 10
Pulse rate (bpm)	66 ± 10
Blood sugar (mg/dl)	98.5 ± 18.5
Total cholesterol (mg/dl)	192.6 ± 20.7
Blood urea nitrogen (mg/dl)	14.0 ± 18.5
Creatinine (mg/dl)	0.69 ± 0.15
PNC (%)	9.5 ± 4.9
PNC(ADP) (%)	20.2 ± 9.9
Δ-PNC	10.7 ± 6.9
P-selectin (%)	13.1 ± 1.7
P-selectin(ADP) (%)	36.6 ± 9.2
Δ-P-selectin	23.6 ± 9.1
hb-PWV (m/s)	5.3 ± 0.9
ba-PWV (m/s)	13.8 ± 3.0

Values are expressed as mean ± s.d.
PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP) − PNC; Δ-P-selectin = P-selectin (ADP) − P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.

Measurement of platelet activation

Sample preparation and measurement of platelet P-selectin (CD62P) and PNC levels were performed according to the method described by Peters *et al*.[24] To minimize platelet activation during blood collection, blood was drawn via a 21 G butterfly needle without the use of a tourniquet. After discarding the first 2 ml of blood, a further 2 ml was collected and immediately added to 200 μl of sodium citrate (3.1%). All antibodies were sourced as follows: Fluorescein isothiocyanate (FITC) labelled IgG1 anti-CD62P from Dainippon Pharmaceutical, Osaka, Japan, phycoerythin (PE) labelled IgG2a anti-CD42b and FITC labelled IgG1 anti-CD11b from Beckman Coulter, Fullerton, CA, USA. As negative controls, FITC-labelled IgG1 (Beckman Coulter, Fullerton, CA, USA) and double-stained (FITC/PE) IgG1 and IgG2a (Dako, High Wycombe, Bucks, UK) irrelevant antibodies were included.

Sample preparation for the measurement of platelet CD62P level: In all, 5 μl of blood was added to a round-bottomed polystyrene tube containing 50 μl of platelet buffer (10 mmol/l HEPES, 145 mmol/l NaCl, 5 mmol/l KCl, 1 mmol/l $MgSO_4$; pH 7.4), and 5 μl of anti-CD62P or control IgG1 antibody. Following gentle suspension, samples were incubated in the dark at room temperature for 20 min without stirring. Then 250 μl of fixative was added and the tubes were incubated for an additional 10 min. The samples were then diluted with 500 μl of buffer and analysed. Flow cytometric analysis was performed within 1 h of fixation.

Sample preparation for the measurement of PNC level: In all, 50 μl of blood was added to a round-bottomed polystyrene tube containing 5 μl of anti-CD42b, and 5 μl of anti-CD11b or isotype control antibodies. Following gentle mixing, samples were incubated in the dark at room temperature for 10 min without stirring. Then 500 μl of fixative was added and the tubes were incubated for additional 10 min. Flow cytometric analysis was performed within 1 h of preparation.

Flow cytometric analysis

Blood samples were analysed in a COULTER EPICS XL Profile Flow Cytometer, Miami, FL, USA, using either single or double fluorochromes. The peak emission intensity of FITC fluorescence was detected at 515 nm and that of phycoerythin fluorescence at 580 nm.

Measurement of platelet CD62P level: After forward and side scatter measurements were made with gain setting in logarithmic mode, platelet-sized events were counted. CD62P-positive platelets were defined as those with a fluorescence intensity exceeding that of 98% of the platelets staining with control antibody.

Measurement of PNC level: After forward and side scatter measurements were made with gain setting in linear mode, neutrophil-sized events were selected. Results were defined as positive when the fluorescence intensity exceeded that of 98% of the isotype-matched (IgG1 and IgG2a) control antibodies staining. Events positive for both CD11b and CD42b were considered to represent PNCs and were expressed as percentages of events with positive CD11b staining.

Evaluation of platelet activation reserve: We evaluated platelet activation reserve, that is, the ability of the platelets to be activated, in a separate experiment. Platelets were activated with 5 μl of adenosine diphosphate (ADP). We also calculated the difference between basal and stimulated states of P-selectin expression (Δ-P-selectin) and PNC level (Δ-PNC) to determine activation reserve.

Statistical analysis

Data are presented as mean±s.d. Univariate linear correlation analysis and multiple regression analysis were used for statistical evaluation. The variables significantly associated with platelet activation on univariate analysis were included in the multiple regression analysis in order to adjust PWV for each variable. Gender differences were evaluated with ANOVA. *P*-values <0.05 were considered to represent statistical significance.

Results

Both ba-PWV and hb-PWV exhibited significant positive correlations with age, systolic, and diastolic blood pressure ($r=0.60$–0.81, $P<0.05$ or <0.01), and pulse rate ($r=0.44$, $P<0.05$, $r=0.65$, <0.01, respectively) (Table 2). For platelet activation and activation reserve, correlations with age were less strong but remained significant ($r=0.36$–0.61, $P<0.05$ or <0.01) with the exception of Δ-P-selectin (not significant, NS), and correlations with systolic and diastolic blood pressure were similar ($r=0.35$–0.53, $P<0.05$ or <0.01) with the exception of P-selectin (NS) (Table 3). However, platelet activation and activation reserve exhibited no significant correlation with pulse rate, blood glucose, total cholesterol, blood urea nitrogen or creatinine. No significant gender-related differences were observed in any of these correlations (Tables 2 and 3).

Table 2 Correlation between PWV and clinical indices

	hb-PWV	*ba-PWV*
Age	0.74**	0.80**
Systolic blood pressure	0.61**	0.81**
Diastolic blood pressure	0.60**	0.74**
Pulse rate	0.44*	0.65**
Blood sugar	−0.05	−0.17
Total cholesterol	−0.03	−0.30
Blood urea nitrogen	−0.32	0.32
Creatinine	0.04	−0.14
Gender		
Male	5.5±1.0	14.1±3.0
Female	5.2±0.8	13.6±3.1

PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP) − PNC; Δ-P-selectin = P-selectin (ADP) − P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.
For parameters from age to creatinine, values are correlation coefficients.
*$P<0.05$.
**$P<0.01$.
For gender, values are mean±s.d., with differences evaluated with ANOVA.

PWVs exhibited significant positive correlations ($r=0.43$–0.74, $P<0.05$ or <0.01) to all indices of platelet activation and reserve (Table 4, Figure 1). When age or blood pressures were adjusted for on multivariate analysis, some indices of platelet activation and reserve were significantly related to PWVs ($r=0.34$–7.67, $P<0.05$ or <0.01). When both age and blood pressures were simultaneously adjusted for, significant correlations remained between platelet activation and reserve and PWVs ($r=2.17$–6.59, $P<0.05$ or <0.01) (Table 4). In other words, although the relationship between PWVs and the indices of platelet activation was strongly affected by age and blood pressure, a significant association remained when these factors were adjusted for.

Discussion

The main finding of this study was that platelet activation and activation reserve were associated with arterial stiffness when analyses were adjusted for age and blood pressure. This suggests that increased arterial stiffness might play an important role in thrombotic events.

Patients with hypertension, cerebrovascular disease, coronary heart disease, diabetes mellitus, and renal failure are recognized to have less arterial compliance than normal subjects.[13–15,17–19] Increased PWV has also been reported to be an independent predictor of cardiovascular events in patients with

Table 3 Correlation between platelet activation and clinical indices

	PNC	*PNC (ADP)*	*Δ-PNC*	*P-selectin*	*P-selectin (ADP)*	*Δ-P-selectin*
Age	0.51**	0.61**	0.52**	0.36*	0.38*	0.32
Systolic blood pressure	0.41*	0.53**	0.48**	0.41*	0.43*	0.35*
Diastolic blood pressure	0.43*	0.49**	0.40*	0.25	0.40*	0.36*
Pulse rate	0.28	0.25	0.16	0.04	0.15	0.15
Blood sugar	0.09	−0.18	−0.31	−0.17	0.13	0.16
Total cholesterol	−0.14	−0.07	0.001	−0.10	−0.13	−0.11
Blood urea nitrogen	−0.01	0.12	0.18	−0.05	0.05	0.06
Creatinine	0.05	−0.13	−0.22	0.04	−0.17	−0.18
Gender:						
male	10.3±5.9	19.7±8.7	9.4±6.9	13.1±1.8	35.5±9.3	22.4±9.0
female	8.8±3.8	20.7±11.4	11.9±6.8	13.0±1.7	37.7±9.2	24.7±9.3

PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)−PNC; Δ-P-selectin = P-selectin (ADP)−P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.
For parameters from age to creatinine, values are correlation coefficients.
*$P<0.05$.
**$P<0.01$.
For gender, values are mean±s.d., with differences evaluated with ANOVA.

Table 4 Relation between platelet activations and PWV

	PNC	*PNC (ADP)*	*Δ-PNC*	*P-selectin*	*P-selectin (ADP)*	*Δ-P-selectin*
Not adjusted						
hb-PWV	0.62**	0.74**	0.63**	0.45**	0.57**	0.50**
ba-PWV	0.59**	0.71**	0.61**	0.47**	0.51**	0.43*
Adjusted for age						
hb-PWV	2.86**	6.95**	4.09*	0.76	6.55**	5.80*
ba-PWV	0.79	2.01**	1.22*	0.28	1.75*	1.47
Adjusted for systolic blood pressure						
hb-PWV	3.20**	7.23**	4.04**	0.59	5.09*	4.50*
ba-PWV	1.21**	2.64**	1.44	0.23	1.48	1.25
Adjusted for diastolic blood pressure						
hb-PWV	3.08**	7.67**	4.59**	0.87*	5.32**	4.46*
ba-PWV	0.97**	2.50**	1.53*	0.34*	1.45*	1.10
Adjusted for age and systolic blood pressure						
hb-PWV	2.80*	6.43**	3.63*	0.58	5.93*	5.35*
ba-PWV	1.08	2.32*	1.24	0.24	1.72	1.48
Adjusted for age and diastolic blood pressure						
hb-PWV	2.63*	6.59	3.97**	0.78	6.06*	5.28*
ba-PWV	0.76	2.12	1.40	0.40	1.66	1.26

PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)−PNC; Δ-P-selectin = P-selectin (ADP)−P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.
'Not adjusted' — values are correlation coefficients between PWVs and indices of platelet activation before adjustment.
*$P<0.05$.
**$P<0.01$.
Other values are regression coefficients between PWVs and indices of platelet activation adjusted for age and/or blood pressures as indicated.
*$P<0.05$.
**$P<0.01$.

hypertension or renal failure, and in elderly subjects.[22,23] The association between increased arterial stiffness and high incidence of cardiovascular events may be explained by the existence of atherosclerosis. Hirai *et al*[25] have demonstrated strong associations between abdominal aortic and carotid arterial stiffness and the degree of coronary artery disease. Popele *et al*[26] recently reported that aortic stiffness as measured by PWV is strongly associated with common carotid intima-media thickness, carotid arterial plaques, and the presence of peripheral arterial disease. Moreover, some population-based studies have demonstrated higher blood pressure, increased age, and male gender to be associated with increased PWV.[16,20,21] Pulse pressure may also relate to arterial stiffness and cardiovas-

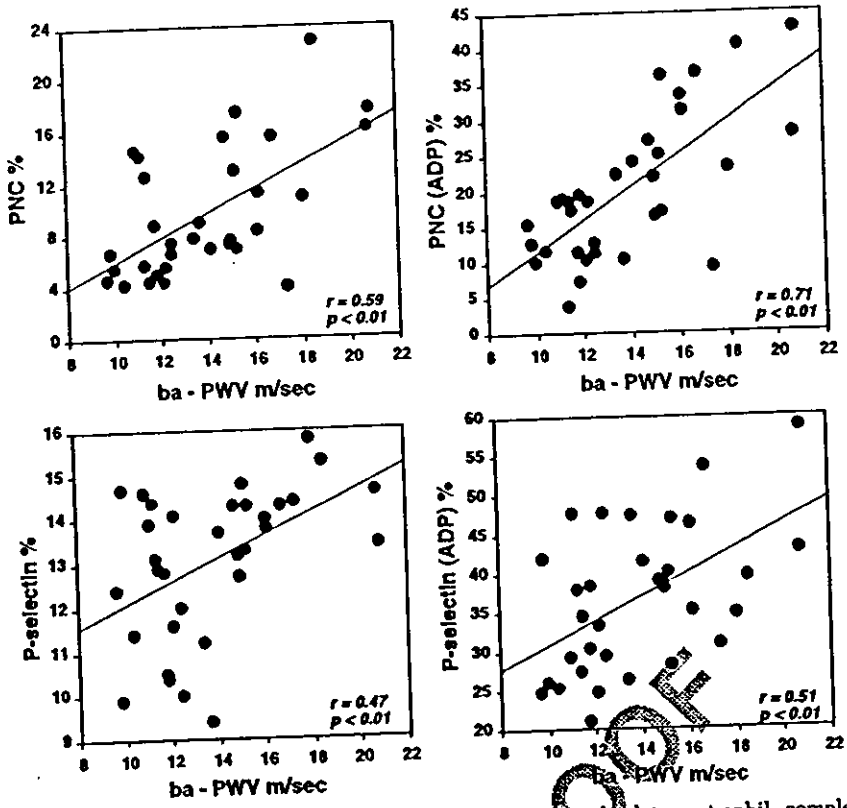

Figure 1 Correlation between ba-PWV and PNC (upper two panels). PNC = platelet neutrophil complexes; ADP = adenosine diphosphate; Δ-PNC = PNC (ADP)–PNC; Δ-P-selectin = P-selectin (ADP)–P-selectin; hb-PWV = heart–brachial pulse wave velocity; ba-PWV = brachial–ankle pulse wave velocity.

cular events, with higher pulse pressure reflecting elevated systolic pressure and reduced diastolic pressure due to increased arterial stiffness. In the present study, significant relationships were observed between PWVs and age, blood pressure, and pulse rate, in accordance with previous studies.

P-selectin is a component of α-granules that is expressed on the platelet surface membrane and released into the plasma upon platelet activation. Although the bulk of circulating soluble P-selectin appears to be platelet derived,[27] the substance is also found in the Weibel–Palade bodies of endothelial cells.[28] Direct measurement of platelet membrane P-selectin is therefore a more sensitive method of assessing platelet activation. In the present study, we evaluated platelet activation by measuring membrane activation markers using flow cytometry with activation-dependent monoclonal antibodies. PNC levels were also measured using the same method. P-selectin levels in our normal subjects aged 49±15 years were 13.1±1.7%; this was higher than that in quoted by other studies, possibly due to the differences in monoclonal antibodies or in sample manipulation.

P-selectin expressed on activated platelets causes formation of PNC. Moreover, platelets and platelet-derived P-selectin play an important role in thrombus growth at the site of atherosclerosis.[2] *In vivo* and *in vitro* studies have shown that shear stress and exposure to atherogenic stimuli, such as oxidization by low-density lipoprotein or cigarette smoking, induce rapid P-selectin-dependent aggregation and accumulation of leukocytes and platelets.[4,5,11] Activated platelets accumulating in thrombi at the site of ruptured atherosclerotic plaques will express CD62P. In clinical studies, P-selectin has been shown to be a marker of platelet activation related to adverse cardiovascular events such as hypertension, coronary artery disease, cerebrovascular disease, and peripheral arterial disease,[6,7,10–12] and also to be a predictor of cardiovascular events.[6,12] PNC, forming as a result of the interaction of platelet P-selectin and neutrophils also promotes platelet activation.[24] This is the first study to demonstrate that P-selectin and PNC were significantly correlated with arterial stiffness evaluated by PWV in normal subjects. In an analysis of four randomized trials, Hebert *et al*[29] showed that aspirin therapy was beneficial in the primary prevention of vascular disease. Higher levels of other membrane markers such as von Willebrand factor receptor are observed in activated platelets, which are affected by aspirin

or ticlopidine.[30] Therefore, our results indicate that, in the normal population, antiplatelet agents may play a role in preventing cardiovascular events through factors other than P-selectin.

Although the exact mechanism accounting for the relationship between platelet activation and arterial stiffness is unknown, it is possible to make the following speculations. When arterial stiffness is raised, shear stress might play an important role in platelet activation. Using cone-plate viscometry,[3] Goto *et al* showed that platelet activation (measured by P-selectin surface expression, von Willebrand factor-mediated platelet aggregation and translocation of GP Ibα) was induced by high shear stress of 10 800/s. Higher arterial stiffness increases blood flow velocity and produces a steep systolic pressure waveform,[31] and it is possible that the resulting increased shear stress could promote platelet activation. Another possible mechanism is that endothelial dysfunction may interact with arterial stiffness and platelet hyperactivity. Kobayashi *et al*[32] showed significant correlation between endothelial dysfunction measured by flow-mediated dilatation and ba-PWV. Platelets are also activated by endothelial dysfunction. On the other hand, activated platelets themselves may cause arterial stiffness via vascular smooth muscle cell growth factors and extracellular matrix modulator released from platelets, that is, PDGF.[33] However, this response also occurs at the site of endothelial injury. Further study is therefore required to clarify whether arterial stiffness causes platelet activation or alternatively whether platelet activation might result in arterial stiffness.

Limitations

Despite the small sample size, it is possible that the broad age range (23–77 years) of our subjects caused outliers in PWV and platelet activation. However, significant correlations were found when age and blood pressure were adjusted for, suggesting that the influence of age did not entirely explain the correlation between PWV and platelet activation. In the present study, ba-PWV was 14.1±3.0 m/s in men and 13.6±3.1 m/s in women; values higher than those reported by Yamashina *et al*.[20] Furthermore, it is not known whether such a relationship between arterial stiffness and platelet activation is found in patients with conditions such as hypertension, diabetes mellitus, coronary heart disease, and stroke. Further studies should be therefore performed in such patients, using larger sample sizes.

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高血圧治療における塩酸ベニジピンとAII受容体拮抗薬併用療法の検討

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要　旨

本態性高血圧患者に対する塩酸ベニジピンとAII受容体拮抗薬併用療法の有用性と安全性を検討した。両剤の併用により良好な降圧効果が得られた。また，降圧による反射性頻脈や検査データの重篤な悪化，投薬を中止するような自覚症状の出現などの副作用も見られず，両剤の併用は高血圧治療において有用かつ安全であると考えられた。さらに，塩酸ベニジピンとAII受容体拮抗薬の投与順序による薬効差は認めず，また，併用するAII受容体拮抗薬の種類による降圧の程度および試験期間中を通じての脈拍にも差はなかった。以上より，塩酸ベニジピンとAII受容体拮抗薬との併用療法は単剤で効果不十分な症例において有用性が高く，安全性にも優れていると考えられた。

Combination Treatment with Benidipine Hydrochloride and Angiotensin II Receptor Blocker for Hypertension

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Ⅰ はじめに

これまで我が国では高血圧患者の治療において，降圧効果が確実であり，禁忌とされる疾患が少ないことから長時間作用型のCa拮抗薬が多く使用されてきた。しかし近年，合併症を伴う高血圧患者を対象とした心・脳血管事故発症に対する研究で，ACE阻害薬がCa拮抗薬より優れているという結果が報告[1)2)]され，またACE阻害薬と降圧効果や臓器保護効果が同等で，咳などの副作用が少なく安全性に優れたAⅡ受容体拮抗薬が第一選択薬として使用される頻度が増加している[3)~6)]。

近年の大規模臨床試験において，十分な降圧が心血管イベントだけでなく脳血管障害のリスクを低下させることが明らかにされ[7)8)]，いくつかの高血圧診断治療指針でも1剤での降圧効果が不十分な場合，降圧の効率を高め，かつ副作用を軽減することができることより，少量多剤併用療法が推奨されている[9)~13)]。Ca拮抗薬は降圧効果が強力であることから，今後は単剤による治療だけでなくACE阻害薬やAⅡ受容体拮抗薬と併用される機会が多くなることが予測される。実際，我が国ではCa拮抗薬やACE阻害薬が汎用されており[3)~6)]，その併用療法の有用性は多く報告[14)~18)]されている。

一方，Ca拮抗薬とAⅡ受容体拮抗薬の併用時の有効性，および安全性についての検討はあまり見られない。そこで今回，ジヒドロピリジン系長時間作用型Ca拮抗薬である塩酸ベニジピンとAⅡ受容体拮抗薬併用における高血圧治療の有用性と安全性を検討した。

Ⅱ 方　　法

1. 対象と方法

収縮期血圧140mmHg以上，拡張期血圧90mmHg以上のどちらか一方を満たす外来通院中の本態性高血圧症患者に2週以上の観察期間を設け，その後，各薬剤の承認された通常投与量，すなわち塩酸ベニジピン4mg/日またはAⅡ受容体拮抗薬（ロサルタン；50mg/日，カンデサルタン；8mg/日，バルサルタン；80mg/日，テルミサルタン；40mg/日）のいずれか一方を4週以上投与した。この時期を1剤投与期とし，血圧，脈拍，体重の測定，血液生化学検査および自覚症状の調査を実施した。1剤投与期の血圧が収縮期血圧140mmHg以上または拡張期血圧90mmHg以上であった患者に，先行投与薬剤の用法・用量を変えず，もう一方のクラスの薬剤を追加投与してこの時期を2剤併用期とした。併用開始後2週以上が経過した時点で1剤投与期と同様に血圧，脈拍，体重の測定，血液生化学検査および自覚症状の調査を実施した。観察期間にすでに投与されていた降圧薬は用法・用量を変更せずに継続した。継続した降圧薬の内訳はCa拮抗薬3例，ACE阻害薬1例，β遮断薬1例，利尿薬1例，α遮断薬1例であった。

重症高血圧（拡張期血圧120mmHg以上）患者，重症心不全患者，過去6カ月以内に発生した急性冠症候群および脳血管疾患患者，HbA_{1c}が7％以上の糖尿病患者は除外した。

2. 検討項目

血圧は座位にて医師または看護師が上腕動脈よりコロトコフ法にて測定し，同時に脈拍数，体重を測定した。

血液学的検査として，赤血球数，ヘモグロビン，ヘマトクリット，白血球数，血小板数を，生化学検査として，総蛋白，アルブミン，血糖，AST，ALT，総ビリルビン，CPK，総コレステロール，中性脂肪，BUN，クレアチニン，尿酸，Na，K，Cl，CRPを測定した。

可能な症例において胸部正面エックス線像を撮影，心胸郭比を計測し，心拡大の指標とした。

自覚症状は，観察期間に認められた症状の推移および試験期間中に新たに出現した症状を患者本人から聞き取った。

3. 副作用

試験期間中に新たに発現し，主治医が投薬を中止する必要があると判定した自・他覚症状を

表1 患 者 背 景

		全 例	AII受容体拮抗薬先行群	塩酸ベニジピン先行群	
n		38	27	11	
年齢（歳）		67±12	65±12	72±11	n.s.
性別（男性）		19	15	4	n.s.
収縮期血圧（mmHg）		168±21	167±19	170±26	n.s.
拡張期血圧（mmHg）		89±12	89±11	91±15	n.s.
脈拍数（/分）		74±10	75±10	73±10	n.s.
1剤投与期間（週）		36.1±36.1	31.3±30.0	39.1±49.7	n.s.
2剤併用期間（週）		36.3±44.4	35.1±34.6	39.1±64.6	n.s.
AII受容体拮抗薬の種類（例数）	・ロサルタン	21	14	7	
	・カンデサルタン	9	7	2	
	・バルサルタン	7	6	1	
	・テルミサルタン	1	0	1	

副作用として扱った。副作用の有無は受診毎に確認した。

また，臨床検査値は，1）正常値から異常値へ明らかに変化したとき，2）観察期間から異常値であったが試験期間中に明らかに増悪したとき，のそれぞれにおいて投与した薬剤との関連性が明確に否定された場合を除いて副作用として扱った。

4. 統計手法

結果は平均値±標準偏差で表した。有意差検定は反復測定ANOVAを用い，post hoc testはScheffeの方法を用いて検討した。有意水準は5％未満とした。

III 結　　果

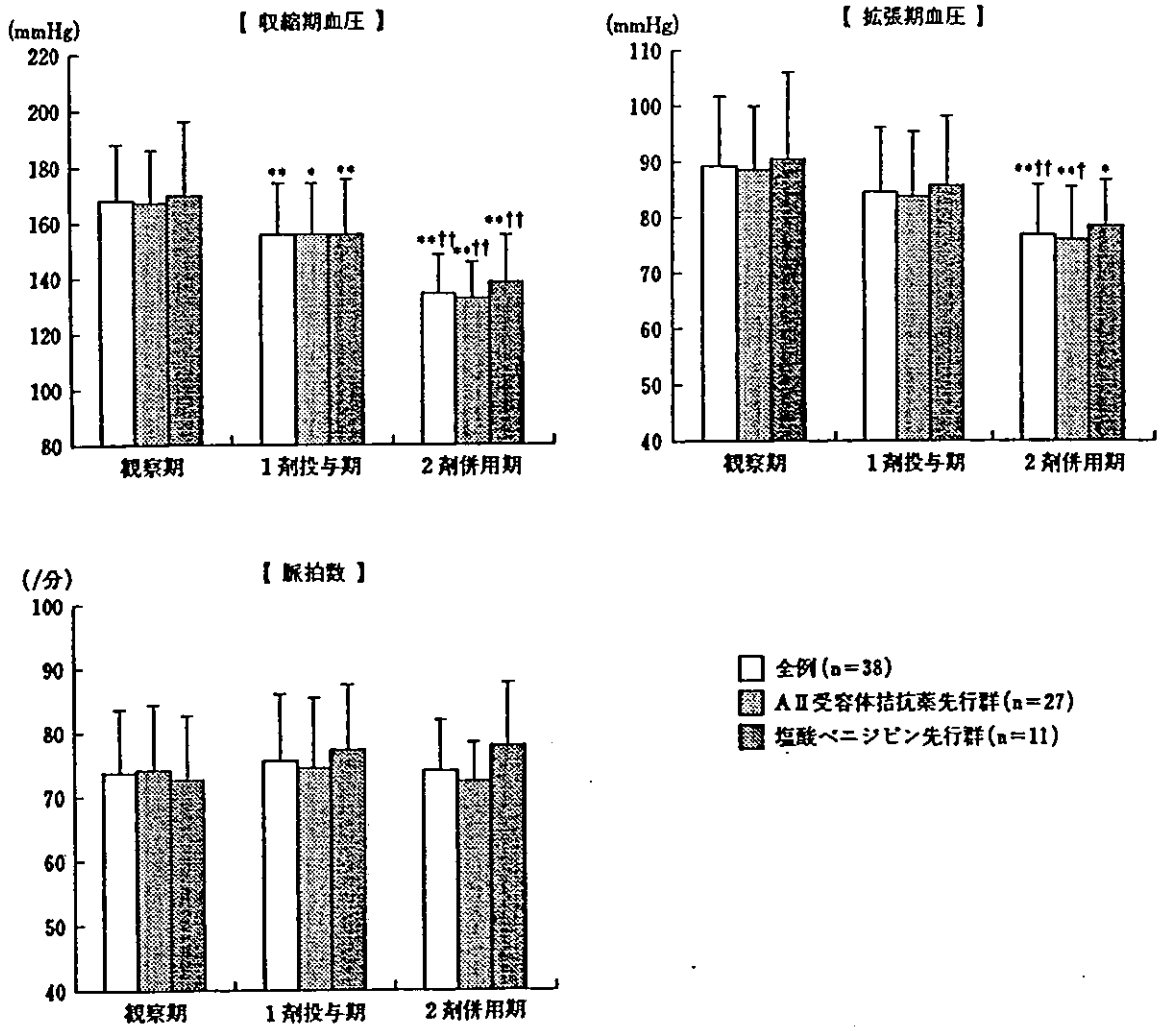
全例，AII受容体拮抗薬先行群および塩酸ベニジピン先行群の背景を表1に示す。AII受容体拮抗薬先行群は27例で，その内訳は，ロサルタン14例，カンデサルタン7例，バルサルタン6例であった。塩酸ベニジピン先行群は11例で，2剤併用期のAII受容体拮抗薬の内訳は，ロサルタン7例，カンデサルタン2例，バルサルタン1例，テルミサルタン1例であった。投与前の年齢，性別，血圧値，脈拍，および1剤投与期間，2剤併用期間はAII受容体拮抗薬先行群と塩酸ベニジピン先行群の間に有意な差は認めなかった。

1. 血圧および脈拍の変化

1）全例での検討

全例での収縮期血圧は観察期間168±21mmHgに対し，1剤投与期155±9mmHg，2剤併用期134±14mmHgといずれも有意に下降した。拡張期血圧は観察期間89±12mmHgに対し，1剤投与期85±11mmHgであったが，2剤併用期には77±9mmHgと有意な下降を認めた（図1）。また，全例での収縮期血圧/拡張期血圧の降圧度は，1剤投与期でそれぞれ−13±16/−5±14mmHgであったが2剤併用によりさらに−22±12/−7±10mmHg降圧し，最終降圧度は−35±17/−12±16mmHgとなった（表2）。2剤併用により血圧が140/90mmHg未満となったものは23例（61％）であった。脈

図1　全例，AII受容体拮抗薬先行群および塩酸ベニジピン先行群の血圧および脈拍の変化



*：p<0.05，**：p<0.01；観察期に対する差　　†：p<0.05，††：p<0.01；1剤投与期に対する差

表2　降圧度と脈拍の変化

		全　例 (n=38)	AII受容体拮抗薬先行群 (n=27)	塩酸ベニジピン先行群 (n=11)	
収縮期血圧 (mmHg)	1剤投与期－観察期	−13±16	−12±15	−15±20	n.s.
	2剤併用期－1剤投与期	−22±12	−23±12	−18±11	n.s.
	2剤併用期－観察期	−35±17	−35±15	−32±21	n.s.
拡張期血圧 (mmHg)	1剤投与期－観察期	−5±14	−5±15	−4±13	n.s.
	2剤併用期－1剤投与期	−7±10	−8±10	−7±12	n.s.
	2剤併用期－観察期	−12±16	−12±16	−12±15	n.s.
脈拍数 (/分)	1剤投与期－観察期	2±8	0.1±6	5±9	n.s.
	2剤併用期－1剤投与期	−1±8	−2±7	0.5±9	n.s.
	2剤併用期－観察期	0.4±10	−2±7	6±14	n.s.

表3　AII受容体拮抗薬種類別の降圧度と脈拍の変化

		ロサルタン (n=21)	カンデサルタン (n=9)	バルサルタン (n=7)	
収縮期血圧（mmHg）	2剤併用期－観察期	−34±16	−34±23	−35±13	n.s.
拡張期血圧（mmHg）	2剤併用期－観察期	−11±19	−15±14	−11±7	n.s.
脈拍数（/分）	2剤併用期－観察期	2±11	−4±10	3±4	n.s.

拍数は，観察期間，1剤投与期，2剤併用期を通じて有意な変化を認めなかった（図1）。

2）AII受容体拮抗薬先行群と塩酸ベニジピン先行群間での検討

AII受容体拮抗薬と塩酸ベニジピンの投与順序による降圧効果の差は認められず，脈拍も両群で有意差は認めなかった（図1，表2）。収縮期血圧/拡張期血圧の1剤投与期における降圧度は塩酸ベニジピン先行群の−15±20/−4±13mmHgに対しAII受容体拮抗薬先行群では−12±15/−5±15mmHgであった。2剤併用期では塩酸ベニジピン先行群はAII受容体拮抗薬追加によりさらに−18±11/−7±12mmHg，AII受容体拮抗薬先行群は塩酸ベニジピン追加により−23±12/−8±10mmHgの降圧が見られ，最終平均降圧度は，それぞれ−32±21/−12±15mmHg，−35±15/−12±16mmHgに達した（表2）。2剤併用により140/90mmHg未満となったものはAII受容体拮抗薬先行群で18例（67%），塩酸ベニジピン先行群で5例（45%）であった。

3）AII受容体拮抗薬種類別での検討

今回テルミサルタンを投与した例は1例であったため除外し，AII受容体拮抗薬と塩酸ベニジピンの投与順序を問わず，ロサルタンを投与した21例，カンデサルタンを投与した9例，バルサルタンを投与した7例で，観察期間および2剤併用後において検討した。観察期間および2剤併用後で，収縮期血圧，拡張期血圧，脈拍ともAII受容体拮抗薬の種類の違いによる有意な差は認めなかった（表3）。

2. 体重，心胸郭比，血液検査データの変化

体重は全例，心胸郭比は17例，血液検査データは34例で解析可能であった。全例（表4），AII受容体拮抗薬先行群，塩酸ベニジピン先行群とも，全期間を通じて体重，心胸郭比，血液生化学検査所見の有意な変化は認めなかった。

3. 自覚症状（図2）

観察期間に自覚症状を有する者は13例（34%）であり，主な自覚症状は頭痛，肩こり，動悸，気分不良，ふらつき等であった。

AII受容体拮抗薬先行群では，観察期間に症状のあった11例中，AII受容体拮抗薬の単独投与で7例が消失し，塩酸ベニジピンの併用投与でさらに2例が消失した。また，このAII受容体拮抗薬の単独投与で症状の消失した7例中1例に塩酸ベニジピンの併用投与で症状を認めた。観察期間に症状のなかった16例中，AII受容体拮抗薬の単独投与で5例に咳，ふらつき等の症状が出現したが，このうち3例は塩酸ベニジピン併用投与で消失した。また，AII受容体拮抗薬の単独投与で症状のなかった11例中，塩酸ベニジピンの併用投与後1例に症状を認めた。

塩酸ベニジピン先行群では，観察期間に症状のあった2例は塩酸ベニジピンの単独投与で消失し，観察期間に症状のなかった9例は2剤併用期まで症状を認めなかった。

全体としては，試験期間中7例に新たに症状が出現した。時期別には1剤投与期に5例，および2剤併用期に2例に発現し，主な症状はふらつき，立ちくらみ，咳，倦怠感等であったが，いずれも軽度であったため投与は継続した。

表4　体重，心胸郭比，血液検査データの変化(全例)

	観　察　期	1剤投与期	2剤併用期	
体重 (kg)	63±11	61±10	62±10	n.s.
心胸郭比 (%)	49±4	48±6	50±6	n.s.
RBC (×10⁴/μL)	450±54	444±61	425±69	n.s.
Hg (g/dL)	14.0±1.6	13.8±1.9	13.3±2.2	n.s.
Ht (%)	41.9±4.5	40.8±5.3	39.5±6.2	n.s.
WBC (×10³/μL)	6.3±1.3	6.2±1.6	6.0±1.4	n.s.
Plt (×10⁴/μL)	22.1±5.6	22.5±5.8	21.7±5.3	n.s.
TP (g/dL)	6.9±0.5	6.8±0.6	6.8±0.8	n.s.
Alb (g/dL)	4.3±0.5	4.1±0.5	4.1±0.6	n.s.
BS (mg/dL)	117±49	135±57	120±41	n.s.
AST (IU/L)	25±12	29±17	27±20	n.s.
ALT (IU/L)	28±25	29±22	26±24	n.s.
T.bil (mg/dL)	0.7±0.3	0.6±0.3	0.7±0.3	n.s.
CPK (IU/L)	104±48	116±76	128±71	n.s.
T.cho (mg/dL)	200±35	201±29	202±32	n.s.
TG (mg/dL)	148±73	165±114	163±94	n.s.
BUN (mg/dL)	15.6±4.7	18.2±5.9	17.6±5.0	n.s.
Cre (mg/dL)	0.8±0.2	0.9±0.3	0.9±0.3	n.s.
UA (mg/dL)	5.3±1.5	5.3±1.2	5.4±1.3	n.s.
Na (mEq/L)	141±3	140±6	142±2	n.s.
K (mEq/L)	4.0±0.4	4.2±0.4	4.2±0.4	n.s.
Cl (mEq/L)	104±4	104±4	104±3	n.s.
CRP (mg/dL)	0.1±0.2	0.2±0.3	0.2±0.1	n.s.

RBC=赤血球数，Hg=ヘモグロビン，Ht=ヘマトクリット，WBC=白血球数，Plt=血小板数，TP=総蛋白，Alb=アルブミン，BS=血糖，T.bil=総ビリルビン，T.cho=総コレステロール，TG=中性脂肪，Cre=クレアチニン，UA=尿酸

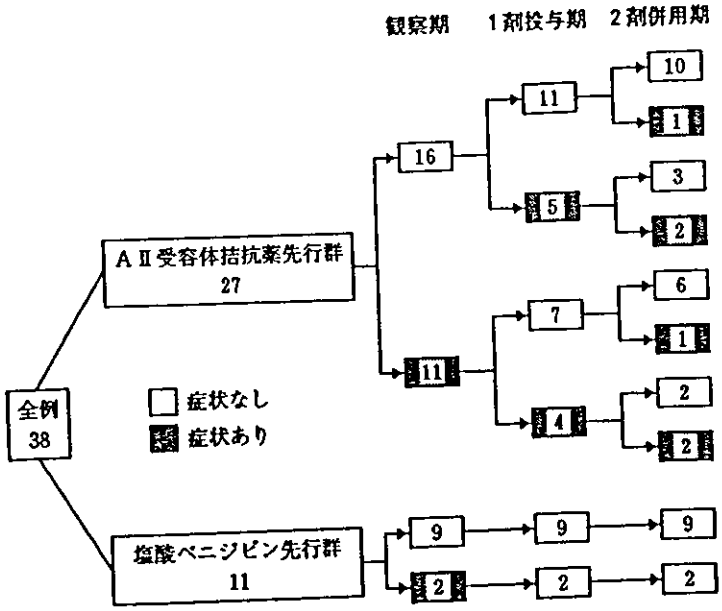
4. 安全性

血液検査所見，体重，心胸郭比とも全期間を通じて有意差は認めなかった（表4）。AII受容体拮抗薬を2剤目として追加投与後，血清クレアチニンが1.0から1.3に上昇した例を1例認めたが，軽度であったため主治医の判断で投薬を継続した。

Ⅳ 考　　察

今回我々は本態性高血圧患者において，塩酸ベニジピンとAII受容体拮抗薬の併用療法の有用性を検討した。両者の併用により良好な降圧効果が得られた。また，降圧による反射性頻脈や検査データの重篤な悪化，投薬を中止するよ

図2　症状の変化



うな自覚症状出現などの副作用もほとんど見られず，両剤の併用は高血圧治療において有用かつ安全であると考えられた。

現在，我が国で最も多く使用されている降圧剤はCa拮抗薬であり，その使用頻度は約70%程度と報告[3)~6)]されている。Ca拮抗薬が多く用いられる理由として，降圧効果が優れていること，副作用が少ないこと，合併症があっても比較的安全に使用できることが挙げられる。また，我が国の心血管イベントは脳血管障害が多く，STONE[19)]，HOT study[20)]，Syst-Eur[21)]等の大規模介入試験により，Ca拮抗薬は脳血管障害に対しての有用性が確立していることも理由の1つである。

最近発表されたALLHAT[7)]やStaessenらのメタ解析[8)]では，降圧薬の作用機序よりも積極的降圧が重要であることが示唆されており，その結果はJNC7など診断治療指針にも反映されている。また，十分な降圧効果を得られない場合には多剤併用が勧められており，診断治療指針による違いはあるにしても，合併症のない症例では利尿薬，Ca拮抗薬，ACE阻害薬，AII受容体拮抗薬，β遮断薬の5剤から，2剤または3剤の併用が推奨されている[9)~13)]。2001年に行われた我が国でのアンケート調査では，これまで用いた2剤併用の組合わせとしては，第一位がCa拮抗薬＋ACE阻害薬（72.4%），第二位がCa拮抗薬＋AII受容体拮抗薬（16.9%）であったが，将来使用する予定の組合わせとしては，第一位がCa拮抗薬＋AII受容体拮抗薬（56.3%），第二位がCa拮抗薬＋ACE阻害薬(24.7%)であった[9)]。諸外国ではすでに，Ca拮抗薬＋ACE阻害薬，利尿薬＋ACE阻害薬，利尿薬＋AII受容体拮抗薬，利尿薬＋β遮断薬などの合剤が販売されており，それらの組み合わせの有用性も報告[12)]されているが，Ca拮抗薬とAII受容体拮抗薬併用についての報告[22)23)]は少なく，塩酸ベニジピンとの併用の評価については今回の報告が初めてである。

今回の検討では，2剤の併用で収縮期血圧－35±17mmHg，拡張期血圧－12±16mmHgの降圧が得られ，最終的に血圧が140/90mmHgの降圧目標値を達成できた症例は全体の61%であった。これは，我が国の高血圧診療におけるこれまでの報告[6)]と同等であるが，JNC7およびESH/ECS2003の診断基準を用いた評価であり，今回の結果を我が国の老年者の高血圧治療ガイドライン[11)]にそって年齢別（70歳未満140/

90mmHg未満，70歳代150/90mmHg未満，80歳以上160/90mmHg未満）に評価すると，70歳未満71%，70歳代67%，80歳以上100%，全体では74%と，十分な降圧が可能であったと考えられた。

ACE阻害薬は，すでに国内外での様々な臨床研究によって生命予後の改善効果が報告[1)24)]されており，心不全など合併症のある症例には積極的に用いるよう推奨されている。AII受容体拮抗薬も降圧効果がCa拮抗薬，ACE阻害薬と同等で，副作用の頻度もCa拮抗薬，ACE阻害薬などより少ないこと[25)]，また，LIFE[26)]，CHARM[27)]などの大規模治験でも心血管イベント抑制効果が示されたことから，合併症のない高血圧症では第一選択薬の1つとして推奨されている[9)~11)13)]。また，これら2剤は弱いながらも交感神経活動を抑制することが報告[28)29)]されており，Ca拮抗薬と併用する場合は，Ca拮抗薬の降圧による反射性交感神経活動性の亢進やレニン-アンギオテンシン系の亢進を抑制することが期待される。

一方，塩酸ベニジピンは，降圧効果と安全性について，単独投与時[30)31)]，および利尿薬・β遮断薬の効果不十分例における併用時[32)]，さらにACE阻害薬の効果不十分例における併用時[16)]について報告されており，我が国においてよく処方されているCa拮抗薬の1つである。今回の検討では，塩酸ベニジピンとAII受容体拮抗薬の併用においてそのどちらを先行投与しても十分な降圧が得られ，脈拍数には有意な変化は認められなかった。1剤投与期ではその降圧効果が十分でなかったため交感神経活性の亢進による反射性の頻脈が見られなかったと考えられるが，2剤併用期においては，降圧に対する交感神経活性の亢進をAII受容体拮抗薬と塩酸ベニジピンが抑制した可能性がある。

Singerら[33)]はCa拮抗薬（ニフェジピン20mg徐放剤）とACE阻害薬（カプトプリル25mg）の併用の検討を報告しているが，単独投与より併用投与の降圧効果が大きく，平均血圧で−22~28mmHg降圧し，投与順序による違いも認められなかったと報告している。しかし，AII受容体拮抗薬はACE阻害薬に比し降圧効果が同等で，咳などの副作用が少なく，認容性が大きい点を考慮すれば，AII受容体拮抗薬の併用の方がより有用である可能性がある。今回の検討でも併用後に重篤な副作用はなく，また65歳以上の高齢者が25例（66%）含まれており，高齢者でも安全に使用できると考えられた。

塩酸ベニジピンと併用したロサルタン，カンデサルタン，バルサルタンはそれぞれ，降圧効果や組織親和性，代謝系への影響に特徴が見られるが[34)35)]，併用後の降圧度や脈拍数，検査値の変化に有意な差を認めなかった。しかし，今回の検討では症例数が少なく，今後症例数を増やしての検討が必要であると考えられる。また，今回の検討では，観察開始時に少数例で他の降圧薬を服用していたが，それらの症例においても重篤な副作用や症状の悪化は認めなかった。2剤で降圧が不十分な症例において，3剤の併用では降圧効果は増大するが，その反面，副作用も増加すると考えられるので，今後十分な症例数で3剤併用の検討が必要であると考えられる。さらに，今回の検討では，投与期間を限定せず評価したため，症例によってばらつきがあるが，これも投与期間をそろえたプロトコールを用い評価する必要がある。

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