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## Evaluation of anti-platelet aggregatory effects of aspirin, cilostazol and ramatroban on platelet-rich plasma and whole blood

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To compare property in anti-platelet effects of aspirin (a cyclooxygenase inhibitor), cilostazol (a phosphodiesterase III inhibitor) and ramatroban (a specific thromboxane A<sub>2</sub> receptor antagonist), we measured human platelet-rich plasma (PRP) aggregation induced by adenosine diphosphate (ADP), collagen and arachidonic acid, and whole blood (WB) aggregation induced by ADP. The release of P-selectin, transforming growth factor-beta 1, and the formation of thromboxane A<sub>2</sub> in response to agonists were also investigated. Inhibitory effects of 100 µmol/l aspirin, 10 µmol/l cilostazol and 1 µmol/l ramatroban on 5 µmol/l ADP-induced PRP aggregation were similar. However, aspirin strongly inhibited thromboxane A<sub>2</sub> formation in response to 5 µmol/l ADP compared with other drugs. Inhibitory effects of 10 µmol/l cilostazol on PRP aggregation and the release of molecules were quite similar in responsiveness induced by the three agonists. Aspirin and cilostazol inhibited platelet aggregation in a concentration-dependent, non-linear fashion, while ramatroban inhibited linearly with increasing concentration. Anti-platelet effects of drugs having different pharmacological mechanisms were demonstrated clearly by measuring PRP aggregation induced by the three agonists, and by measuring WB

aggregation that most probably reflects not only platelet-platelet interactions, but also platelet-leukocyte interactions, as well as the release of intraplatelet molecules. *Blood Coagul Fibrinolysis* 15:157-167 © 2004 Lippincott Williams & Wilkins.

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**Keywords:** whole blood aggregation, platelet-rich plasma, aspirin, cilostazol, ramatroban

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### Introduction

Anti-platelet drugs are administered to prevent the formation of thrombus due to platelet activation in patients with myocardial infarction, thrombotic strokes and peripheral vascular disease [1,2]. It is necessary to manage the effects of anti-platelet drugs to within an appropriate therapeutic range to protect patients from the formation of thrombus and bleeding. Measurement of platelet aggregation has been widely carried out to assess platelet function and the effects of anti-platelet drugs. By far the most common method to detect platelet aggregation is by measuring light transmission of platelet-rich plasma (PRP) [3], while another method to detect platelet aggregates in PRP is a light-scattering method based on a particle counting [4,5]. There are also some methods using whole blood (WB) such as electrical impedance aggregometry [6,7], measurement of closure time of an artificial vessel with a bioactive

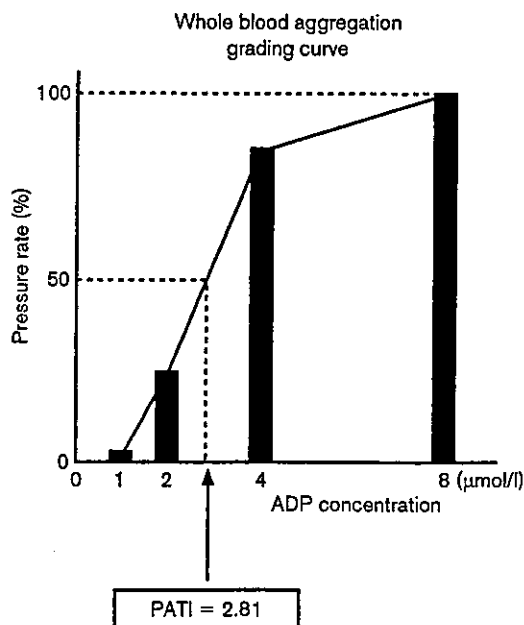
membrane having a microscopic aperture [3,8], light absorbance measurement of blood samples based on agglutination of platelets to fibrinogen-coated beads [3,9,10], and a screen filtration pressure method that measures platelet aggregation in terms of increasing resistance of WB sample flow through a microsieve [11,12]. Notably, the screen filtration pressure method has not been widely utilized to date, largely due to the difficulty of measurement of screen filtration pressure, causing problems with reproducibility and difficulty in the measurement of platelet aggregation. Platelet aggregation is probably mediated by platelet-leukocyte interactions regulated by chemical mediators from leukocytes, erythrocytes and other cells [13,14]. Therefore, it is believed that platelet aggregation in WB could possibly reflect physiological conditions more accurately than aggregation in PRP. Recently, a new type of WB aggregometer with a screen filtration

diameter area. The final platelet aggregation pressure of each reaction tube was determined as the pressure rate of a pressure sensor connected to the syringe. The pressure rate was standardized by a grading curve; 1, 2, 4 and 8  $\mu\text{mol/l}$  ADP were plotted on the  $x$  axis and the pressure rates (%) were plotted on the  $y$  axis. Figure 1 shows a representative analysis of platelet aggregation by the WB aggregometer using a grading curve. The concentration of ADP inducing a 50% pressure rate was calculated and indicated as the platelet aggregatory threshold index (PATI). PATI values were used to evaluate WB aggregation.

#### Time course change in the PATI after blood collection and reproducibility in WB aggregation

To investigate changes in the PATI after blood collection over time, WB aggregation was measured at 5, 15, 30, 60 and 120 min after blood collection in eight healthy volunteers. The reproducibility of WB aggregation was also examined in blood samples. At 60 min after blood collection, the PATI values determined from WB on one day were compared with values from another day when the same measurements were performed.

Fig. 1



A representative analysis of whole blood aggregation using a grading curve. The  $x$  axis is adenosine diphosphate (ADP) concentration, and the  $y$  axis is pressure rate (%). The concentration of ADP inducing 50% pressure rate was calculated and indicated as a platelet aggregatory threshold index value. Whole blood aggregation was measured at 60 min after blood collection. PATI, platelet aggregatory threshold index.

#### Estimation of drug effects on WB aggregation and its comparison with PRP aggregation

At 60 min after blood collection, the working solutions of aspirin, cilostazol and ramatroban were added to WB, followed by incubation for 3 min. Drug-treated WB was stimulated by ADP and WB aggregation was measured as already described. In addition, PATI values in PRP aggregation were also calculated from light transmission 5 min after ADP addition in a similar manner to WB (Fig. 2), and were compared with those in WB aggregation.

#### Co-existence of platelets and leukocytes on the microsieve

To investigate the co-existence of platelets and leukocytes on the microsieve after WB aggregometry, staining of platelets and leukocytes was performed by use of dual-colour immunofluorescence. Erythrocytes on the microsieve were lysed with lysis buffer containing  $\text{NH}_4\text{Cl}$ ,  $\text{NaHCO}_3$  and  $\text{EDTA}2\text{Na}$ , and then were fixed with commercially available solution (Cell Fix; Becton Dickinson, San Jose, California, USA). After being washed three times, the microsieves were pre-incubated with 5% bovine serum albumin in phosphate-buffered saline (pH 7.4) at room temperature. After removing the solution, the microsieves were incubated with saturating concentrations of PE-conjugated anti-CD42b and FITC-conjugated anti-CD45 in a total volume of 500  $\mu\text{l}$  phosphate-buffered saline containing 2% bovine serum albumin for 4 h at 4°C in the dark, then washed five times with phosphate-buffered saline. Images of the immunofluorescent samples were obtained using a CSU-10 confocal laser scanning unit (Yokogawa Electric Co., Tokyo, Japan) coupled to an IX90 inverted microscope with UPlanAPO  $\times 20$  objective lens (Olympus Optical Co., Tokyo, Japan) and a C5810-01 colour chilled 3CCD camera (Hamamatsu Photonics K. K., Hamamatsu, Japan) [30,31].

#### Statistical analysis

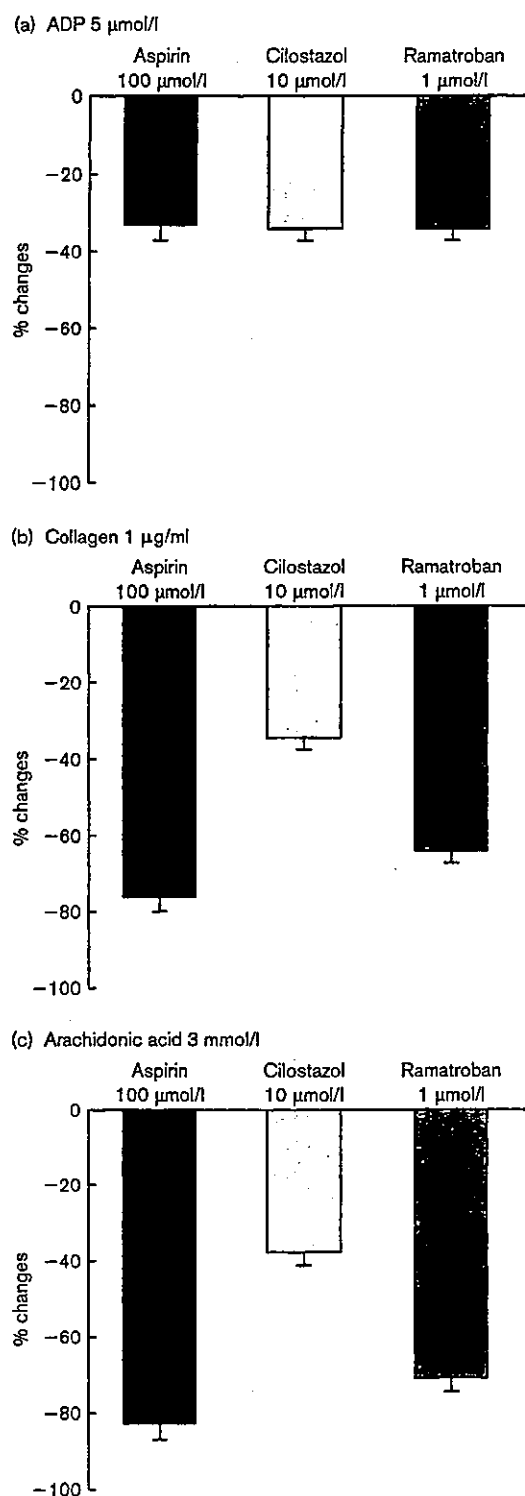
The data are expressed as means  $\pm$  standard deviation. Statistical analysis was performed by a one-way analysis of variance, which was followed by Fisher's protected least significant difference test. Relationships between independent variables were assessed by Pearson's correlation. Differences were considered to be significant at  $P < 0.05$ . StatView J-4.5 software (Abacus Concepts Inc., Berkeley, California, USA) was employed for all calculations.

## Results

#### Inhibitory effects of aspirin, cilostazol and ramatroban on PRP aggregation

MA values from control PRP in response to ADP (1 and 5  $\mu\text{mol/l}$ ), collagen (1  $\mu\text{g/ml}$ ) and arachidonic acid (3  $\text{mmol/l}$ ) are presented in Table 1. When control PRP was stimulated by 1  $\mu\text{mol/l}$  ADP, primary aggregation was induced in all specimens, with MAs of 40% or less,

Fig. 3



Inhibitory effects of aspirin, cilostazol and ramatroban on platelet-rich plasma (PRP) aggregation induced by (a) adenosine diphosphate (ADP) (5 µmol/l), (b) collagen (1 µg/ml) and (c) arachidonic acid (3 mmol/l). PRP samples were pre-incubated with drugs for 3 min prior to the addition of agonists. Effects of drugs were evaluated as percentage changes in maximum aggregation against vehicle-treated samples. Data represent mean  $\pm$  standard deviation ( $n = 8$ ).

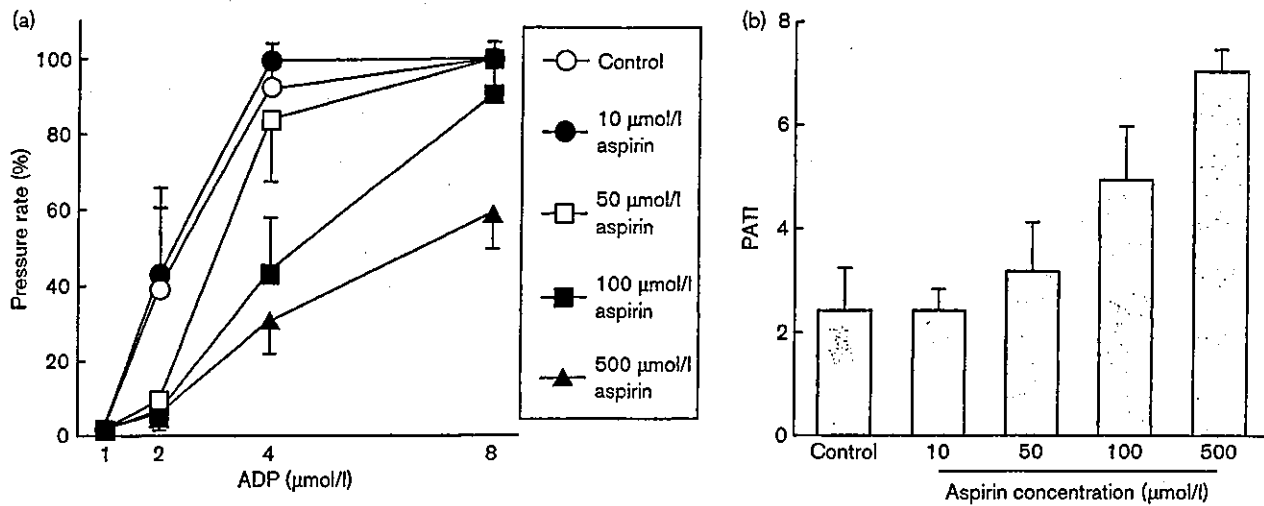
was stimulated by 1 µmol/l ADP, sP-selectin and TGF-β1 levels increased significantly compared with their circulating plasma levels ( $P < 0.05$ ). TXB<sub>2</sub> also showed high levels, although no difference was found between the levels in response to 1 µmol/l ADP and the levels in circulating plasma. The levels of sP-selectin, TGF-β1 and TXB<sub>2</sub> in response to 5 µmol/l ADP, collagen (1 µg/ml) and arachidonic acid (3 mmol/l) were significantly higher than those in response to 1 µmol/l ADP ( $P < 0.001$ ). Furthermore, TXB<sub>2</sub> levels in response to arachidonic acid markedly increased compared with those in response to 5 µmol/l ADP ( $P < 0.001$ ).

The inhibitory effects of aspirin, cilostazol and ramatroban on the release of P-selectin, TGF-β1 and TXA<sub>2</sub> from platelets in response to ADP (5 µmol/l), collagen and arachidonic acid are shown in Figure 4. When PRP was stimulated by ADP (5 µmol/l), 100 µmol/l aspirin significantly inhibited the release of TXA<sub>2</sub> compared with that of P-selectin and TGF-β1. In contrast, inhibitory effects of 10 µmol/l cilostazol and 1 µmol/l ramatroban on the release of TXA<sub>2</sub> in response to ADP (5 µmol/l) were similar to those on the release of P-selectin and TGF-β1. Aspirin and ramatroban markedly inhibited the release of P-selectin, TGF-β1 and TXA<sub>2</sub> to a similar degree in response to collagen and arachidonic acid. However, 10 µmol/l cilostazol inhibited the release of the three molecules in response to ADP, collagen and arachidonic acid to a similar degree.

#### Time-dependent change after blood collection and reproducibility in WB aggregation

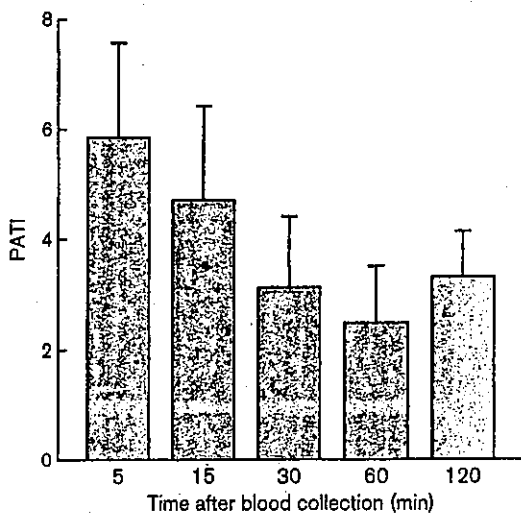
In order to investigate the relationship between ADP concentration and pressure rate, WB aggregation induced by 1, 2, 4 and 8 µmol/l ADP was measured. As shown in Figure 5a, when control WB and aspirin-treated (10, 50, 100 and 500 µmol/l) WB were stimulated by ADP, the pressure rates in all samples increased depending on ADP concentrations. These data were presented as the dose-response plots for aspirin concentrations in Figure 5b. PATI values elevated with the increase in aspirin concentration. Figure 6 shows time-dependent changes in the PATI values after blood collection. PATI values immediately after blood collection (5 min) were  $5.68 \pm 1.71$ , and decreased gradually until 60 min after blood collection, and then increased slightly after 120 min. The following experiments to evaluate WB aggregation were performed using blood samples 60 min after collection. In the examination of reproducibility between two different sample days, the mean values of PATI from the same volunteers were  $2.63 \pm 0.65$  and  $2.52 \pm 0.84$  ( $n = 8$ ), respectively, and no significant difference in PATI values between the two days was observed. While platelet counts from eight volunteers ranged from  $17.5 \times 10^4$  to  $31.2 \times 10^4$  cells/µl [mean platelet count,  $(25.0 \pm 5.0) \times 10^4$  cells/µl], PATI values were in

Fig. 5



Relationship between adenosine diphosphate (ADP) concentration and pressure rate (%) in whole blood aggregation. Whole blood was pre-incubated with or without aspirin for 3 min prior to the addition of ADP (1, 2, 4 and 8 μmol/l). Whole blood aggregation was measured at 60 min after blood collection. Data represent mean ± standard deviation ( $n = 8$ ). PATI, platelet aggregatory threshold index.

Fig. 6



Time-dependent changes in platelet aggregatory threshold index (PATI) values after blood collection. Whole blood was stimulated by adenosine diphosphate (ADP) (1, 2, 4 and 8 μmol/l), and whole blood aggregation was measured at 5, 15, 30, 60 and 120 min after blood collection and PATI values were obtained. Data represent mean ± standard deviation ( $n = 8$ ).

least three receptors; the P2X receptor that mediates rapid Ca ion influx, the P2Y1 receptor coupled to Gq-protein that stimulates phospholipase C (PLC) and induces shape change, and the P2TAC receptor coupled to the Gi-protein that inhibits adenylyl cyclase [32]. Since aspirin inhibited ADP-induced PRP aggrega-

tion to a certain degree in the present study, aspirin, a cyclooxygenase inhibitor, may cause the inhibition of PRP aggregation in response to ADP through the P2Y1 receptor. The effects of aspirin on platelet aggregation in response to collagen may be due to the inhibition of platelet activation via glycoprotein (GP) Ia/IIa (the  $\alpha_2\beta_1$  integrin), CD36 (GPIV, also known as GPIIb), or GPVI, which are collagen receptors and lead to PLC $\gamma$ 2 tyrosine phosphorylation and bring about TXA<sub>2</sub> formation [33]. GPIV may accelerate the rate of adhesion once platelets establish contact with collagen fibres via GPVI and GPIa/IIa [34]. Aspirin markedly inhibits PRP aggregation in response to arachidonic acid, because arachidonic acid is utilized in TXA<sub>2</sub> formation.

Next, we measured the levels of sP-selectin, TGF- $\beta$ 1 and TXB<sub>2</sub> in response to ADP, collagen and arachidonic acid. Aspirin markedly decreased TXB<sub>2</sub> levels compared with levels of sP-selectin and TGF- $\beta$ 1 in response to ADP. In contrast, the rates of decrease of TXB<sub>2</sub> by cilostazol and ramatroban were similar to those of sP-selectin and TGF- $\beta$ 1 in response to ADP. Furthermore, aspirin and ramatroban strongly inhibited the release of P-selectin, TGF- $\beta$ 1 and TXB<sub>2</sub> in response to collagen and arachidonic acid compared with the response induced by ADP. These results suggest that aspirin may inhibit not only TXA<sub>2</sub> formation via cyclooxygenase, but also could inhibit the release reaction of TXA<sub>2</sub> via activation of PLC. Stimulation of platelets by collagen and arachidonic acid induces TXA<sub>2</sub> production and the release reaction of intraplatelet substances. Ramatroban inhibits TXA<sub>2</sub> binding to the TXA<sub>2</sub> receptor on the surface of plate-

**Table 2** Correlations between pressure rate in whole blood aggregometry and other independent variables from platelet-rich plasma (PRP) aggregometry

	Whole blood:PRP	Coefficients (R)	P value
Aspirin-treated samples	Pressure rate:MA	0.849	< 0.001
	Pressure rate:sP-selectin	0.897	< 0.001
	Pressure rate:TGF- $\beta$ 1	0.920	< 0.001
	Pressure rate:TXB <sub>2</sub>	0.737	< 0.001
	PATI:PATI	0.920	< 0.001
Cilostazol-treated samples	Pressure rate:MA	0.781	< 0.001
	Pressure rate:sP-selectin	0.823	< 0.001
	Pressure rate:TGF- $\beta$ 1	0.766	< 0.001
	Pressure rate:TXB <sub>2</sub>	0.785	< 0.001
	PATI:PATI	0.828	< 0.001
Ramatroban-treated samples	Pressure rate:MA	0.638	< 0.001
	Pressure rate:sP-selectin	0.768	< 0.001
	Pressure rate:TGF- $\beta$ 1	0.774	< 0.001
	Pressure rate:TXB <sub>2</sub>	0.802	< 0.001
	PATI:PATI	0.894	< 0.001

MA, percentage maximum aggregation; sP-selectin, soluble P-selectin; TGF- $\beta$ 1, transforming growth factor-beta 1; TXB<sub>2</sub>, thromboxane B<sub>2</sub>; PATI, platelet aggregatory threshold index.

activation through a system linked to PLC [35]. Cilostazol inhibited the release of all the molecules in response to ADP, collagen and arachidonic acid in a similar fashion. These results may be due to the difference in pharmacological mechanisms between aspirin (a cyclooxygenase inhibitor), cilostazol (a phosphodiesterase III inhibitor) and ramatroban (a specific TXA<sub>2</sub> receptor antagonist). Different signal pathways in platelets most surely play important roles in these events.

Third, we examined the effects of these drugs on WB aggregation induced by ADP (1, 2, 4 and 8  $\mu$ mol/l) using the newly developed WB aggregometer with the screen filtration pressure method, and the results were compared with those from PRP aggregation induced by the same agonist. To measure WB aggregation, some devices such as the PFA-100 [3,8], and the Ultegra Platelet Analyzer [3,9,10] have been developed. In the present study, a WB aggregometer with a microsieve made of nickel, 3.7 mm in diameter, with 300 openings/1 mm diameter area was used [15]. The pressure rate (%) of WB through the microsieve after stimulation by ADP (1, 2, 4 and 8  $\mu$ mol/l) was measured. Although we did not compare data from this device with another WB aggregometer, it is reported that platelet aggregation data from the PFA-100 and the Ultegra correlated well to data from a four-channel Chrono-log Lumi-Aggregometer using PRP [10]. We obtained the result that the pressure rate (%) of WB through the microsieves and maximum aggregation rate (%) of PRP correlated closely. In addition, there were close correlations between PATI values from WB aggregation and PRP aggregation, and also between the pressure rate (%) of WB and the levels of molecules released from platelets. Therefore, we believe that data from the newly developed WB aggregometer bear a striking

resemblance to other devices such as the PFA-100 and the Ultegra. Recently, the effects of anti-platelet drugs were successfully evaluated using the WB aggregometer with the screen filtration pressure method [16]. In the present study, we confirmed a high reproducibility of WB aggregation by this apparatus. On the other hand, the PATI value (which represents the concentration of ADP inducing a 50% pressure rate) was highest at 5 min after blood collection and then gradually decreased up to 60 min time, in accordance with previous reports [15,16]. Thus, the data from the WB aggregometer were variable over the time period studied. Although the reason why the PATI value varies depending on time has not yet been clarified, it is possible that a change in the responsiveness of receptors on blood cells or unstable anti-thrombotic physiological substances such as prostacyclin may be contributing factors [15]. Therefore, in clinical laboratories, it is necessary to decide on the time after blood collection determining WB aggregability in order to obtain accurate results. In addition, it is possible to use this device as a rapid bedside test by measuring immediately after blood collection.

In fact, clinical research on subjects who had taken aspirin for angina pectoris is now being conducted. Addition of ADP to WB samples collected from the patients was performed punctually 60 min after drawing blood. In our unpublished data, PATI values in WB aggregation on the seventh day after cessation of taking aspirin to prevent extra bleeding during surgery of coronary artery bypass grafting were lower than those in the aspirin-taking period ( $2.19 \pm 1.33$  versus  $4.81 \pm 2.03$ ). These results suggest that the WB analyser with screen filtration pressure method is a useful instrument even in clinical aspects to evaluate the anti-platelet effects of drugs, as well as other devices.

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## Increased Expression of Humanin Peptide in Diffuse Type Pigmented Villonodular Synovitis. Implication of its Mitochondrial Abnormality

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Increased Expression of Humanin Peptide in Diffuse Type Pigmented Villonodular Synovitis  
Implication of its Mitochondrial Abnormality (Extended Report)

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Key Words; Pigmented Villonodular Synovitis, Mitochondria, Humanin

**Objectives:** To define the pathogenesis of pigmented villonodular synovitis (PVNS), we searched for highly expressed genes in primary synovial cells from PVNS patients.

**Methods:** Using a combination of subtraction cloning and Southern colony hybridization, highly expressed genes in PVNS were detected in the comparison with rheumatoid synovial cells.

Northern hybridization was performed to confirm the differential expression of the humanin gene in PVNS. The expression of the humanin peptide was analyzed by Western blotting and immunohistochemistry. Electron microscopic immunohistochemistry was performed to investigate the distribution of this peptide within the cell.

**Results:** Sixty eight highly expressed genes were identified in PVNS. Humanin genes were strongly expressed in diffuse type PVNS, but were barely detected in nodular type PVNS, RA or OA. The presence of humanin peptide was identified in synovium from diffuse type PVNS and most of the positive cells were distributed in the deep layer of the synovial tissue. Double staining with anti-humanin and anti-heat shock protein 60 showed that humanin was expressed mainly in mitochondria. Electron microscopy revealed immunolocalization of this peptide predominantly around dense iron deposits within the siderosome.

**Conclusions:** Increased expression of the humanin peptide in mitochondria and siderosomes is the characteristic of synovial cells from diffuse type PVNS. Humanin is an anti-apoptotic peptide which is known to be encoded in the mitochondrial genome. Present findings suggest that mitochondrial dysfunction may be primary in the pathogenesis of diffuse type PVNS and that the humanin peptide may be involved in the neoplastic process in this form of PVNS.

## Introduction

Pigmented Villonodular Synovitis (PVNS) is classified as an uncommon idiopathic proliferative synovial process. (1,2) It can exist in a localized form within a joint but more commonly occurs as a diffuse form where the entire synovium of a joint is affected. (3,4,5,6,7) The exact etiology of PVNS is still unknown.(8,9,10) Previous experimental and epidemiologic studies have suggested that PVNS is a reactive process involving a chronic inflammatory response. (11,12) However, recent studies showing the capacity of these lesions for autonomous growth and the potential for recurrence have suggested involvement of a neoplastic process. (13) The neoplastic hypothesis has been further supported by studies suggesting that heterogeneous proliferating cells, such as fibroblasts, histiocytes, multinuclear cells and chronic inflammatory cells, might be neoplastic, with other cell types being reactive in nature. (14,15)

Histologically, PVNS is composed of proliferating mononuclear cells, with frequent giant cells, and intracellular and extracellular iron deposits. These iron deposits are observed as membrane-bound particles in siderosomes. Interestingly, Schumacher et al.(16) and Ghadially et al.(18) reported that the siderosome fuses with mitochondria in deep synovial cells from PVNS patients. Moreover, abundant mitochondria throughout the cytoplasm were observed in dispersed stromal cells containing electron-dense inclusions and in giant cells.(16,18)

In the present study, we searched for highly expressed genes in primary synovial cells from PVNS patients compared to those from patients with RA. We supposed that the comparison of synovial cells from PVNS with those from RA, which are composed of chronic inflammatory

cells, would identify the distinct nature of PVNS and define this proliferative process more precisely. Ribosomal RNA (rRNA) with poly A tail encoded by mitochondrial genes were highly expressed in PVNS. Among these genes, humanin has been reported to act as an oncopeptide or as an anti-apoptotic factor against Bax (Bcl2-associated X protein), which is an apoptosis-inducing protein. (19,20) However, little is known about the pathological role of humanin in diseases other than Alzheimer's disease.

We report here that the expression of humanin peptide is increased strongly in diffuse type PVNS compared to other arthrides and it is abundant in mitochondria and siderosomes of synovial cells from PVNS.

## Methods

### **Synovial tissue preparation and RNA extraction.**

Synovial biopsy specimens were obtained during surgery from 6 patients with PVNS, 3 with RA and 3 with osteoarthritis (OA). These lesions were subtyped into two types (diffuse or nodular type) according to locations (intra-versus extraarticular) and pathological growth patterns, which reflected clinical characteristics and biological behavior. (13)

The RA patients met the criteria of the 1987 American College of Rheumatology. The tissue was cultured in IMDM with collagenase V(1mg/1ml medium) for 40 minutes and cells were harvested through mesh and gathered by centrifugation. Total cellular RNA was extracted using AGPC methods.(21) Equal aliquots were then electrophoresed on 1% agarose gels

stained with ethidium bromide to compare large and small rRNA qualitatively and to exclude degradation. Poly A<sup>+</sup> RNA was purified from total RNA using the First Track kit (Invitrogen).

**Double-stranded cDNA synthesis and subtraction cloning.** One µg of total RNA sample was used to synthesize full-length double-stranded cDNA using a SMART PCR cDNA Synthesis Kit (Clontech). Subtraction cloning was performed using a PCR-Select™ cDNA Subtraction Kit (Clontech). Equal amounts of double-stranded cDNAs from two patients with PVNS (diffuse type / lane 1, nodular type / lane 2 in Fig.2) were used as tester cDNAs and equal amounts of double-stranded cDNAs from 3 RA patients were used as driver cDNAs. PCR using CD163 primers was performed to estimate the efficiency of subtraction and the expected decrease in CD163 abundance in the subtracted sample was observed (data not shown).

#### **Southern colony hybridization.**

Subtracted cDNAs were ligated to TOPO vector (Invitrogen) and transformed into DH10B cells (Invitrogen) by electroporation. After blue-white selection with X-gal containing LB plates, white colonies were cultured overnight with 150 µl LB medium in sterile 1.5 ml tubes and centrifuged for 2 min at 12000 g, and the pellet was resuspended in 10 µl LB medium. The medium was mixed completely and 2 µl were dotted on a nylon membrane for Southern hybridization. SMART double-stranded cDNA was labeled with [<sup>32</sup>P]-dCTP by random priming (Stratagene). Membranes were hybridized in aqueous solution (5 x SSC, Denhardt's solution, 0.1% SDS, 10 mg salmon sperm DNA) overnight at 65°C. After washing at 65°C for 1 hour in 0.1 x SSC, 0.1% SDS, the membranes were exposed to X-ray film (Eastman

Kodak Co.) with an intensifying screen at  $-80^{\circ}\text{C}$ . Quantitation of cDNA was performed by scanning with a BASS 1000 Densitometer (Fuji film), and normalization against GAPDH cDNA hybridized subsequently on the same blots.

**DNA sequencing.** Sixty eight cDNAs from differentially expressed clones were amplified with M13 reverse (5' CAGGAAACAGCTATGAC3') primers using thermal cycling conditions ( $96^{\circ}\text{C}$  for 30 seconds,  $50^{\circ}\text{C}$  for 15 seconds,  $60^{\circ}\text{C}$  for 4 minutes for 25 cycles). The cDNAs were purified and sequenced using the ABI PRISM<sup>TM</sup> Dye Terminator Cycle Sequencing Ready Reaction Kit with Template Suppression Reagent (ABI PRISM<sup>TM</sup>). DNA sequences were analyzed using DNASIS software and compared to sequences in GeneBank (National Center for Biotechnology Information, Bethesda, MD).

**Northern hybridization.** Poly A<sup>+</sup> RNA (168 ng) samples of the synovium from 5 PVS, 3 RA and 3 OA patients were loaded and fractionated through 1.0 % agarose gels and transferred to Hybond<sup>TM</sup>-N+ nylon transfer membrane (Amersham). Purified human cDNA (40 ng) was labeled with [<sup>32</sup>P-dCTP] by random priming and applied to the membrane for hybridization in aqueous solution (5 x SCC, Denhardt's solution, 0.1% SDS, 10mg salmon sperm DNA, 50% formamide) overnight at  $42^{\circ}\text{C}$ . After washing at  $42^{\circ}\text{C}$  for 1 hour in 0.1 x SSC, 0.1% SDS, the membranes were exposed to X-ray film (Eastman Kodak Co.) with an intensifying screen at  $-80^{\circ}\text{C}$ .

#### **Semiquantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).**

Total RNA (2.5  $\mu\text{g}$ ) from 5 patients with PVNS, 3 with RA and 2 with OA were used for cDNA

synthesis with oligo(dT)<sub>12-18</sub> as template primer using M-MuLV reverse transcriptase. The reaction was conducted in a final volume of 50 µl containing 1 ml of the transcribed cDNA probe, 200 µM of each dNTP, 1 X PCR buffer including 1.5 mM MgCl<sub>2</sub> (Takara Biomedical), 0.4 µM forward and reverse primers, and 2.5U Taq polymerase (Takara). All amplicons were amplified simultaneously with GAPDH as internal standard. The respective primer pairs were for cytochrome c (forward; 5'-GCATAAACAAACATAAGCTTCTGA-3', reverse; 5'-CAGCAGATCATTTCATATTGCTT-3'), for ATPase (forward; 5'-TCTCATCAACAACCGACTAATCA-3', reverse; 5'-GATAAGTGTAGAGGGAAGGTAA-3'), for NADH dehydrogenase (forward; 5'-TTTACTCAATCCTCTGATCAGGG-3', reverse; 5'-CGAATTCATAAGAACAGGGAGGT-3'), and for cytochrome b (forward; 5'-AATTACAACTTACTATCCGCCA-3', reverse; 5'-TGGGCGAAATATTATGCTTTTGTT-3'). The reactions were incubated for 3 min at 94°C, followed by 32 cycles of denaturation for 1 min at 94°C, annealing for 1.5 min at 52°C, and extension for 1 min at 72°C.

#### **Cell and tissue processing for light microscope and immunohistochemistry.**

To isolate synovial cells, the deep layers of synovium from diffuse type of PVNS were cultured in IMDM with collagenase V (1mg/1ml medium) for 20 minutes and cells were harvested through mesh and gathered by centrifugation. These synovial cells were cultured in IMDM with 10% FBS for 4 hours and fixed with 10% buffered formaldehyde at room temperature for



10 min, rinsed with PBS. Formalin-fixed tissue sections were also used for immunostaining. A rabbit polyclonal anti-humanin antibody was synthesized and purified on an affinity column and dissolved into PBS (0.9% NaCl, 0.02M phosphate buffer, pH7.0). The IgG concentration was analyzed using the Protein Assay kit (Bio-Rad). Immunostaining was performed as previously described.(22,23) Briefly, cells and sections were fixed with 4% formaldehyde in PBS. Following a rinse with PBS, membrane perforation treatment was performed with 95% ethanol/ 5% acetic acid for 10 minutes. After washing with PBS and blocking by incubation with 1% BSA, excess BSA was then removed and the cells were incubated with anti-humanin antibodies overnight at 4°C. After rinsing, Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Inc.) was applied as a secondary antibody for 60 min. Immunofluorescence was detected using a CSU-10 confocal laser scanning unit (Yokogawa Electric Co.), coupled to an IX90 inverted microscope with UPlanAPOX20 objective lens (Olympus Potical Co.), and C5810-01 color chilled 3CCD camera(Hamamatsu Photonics, K.K.). For double staining, anti-humanin antibody and anti-HSP60 antibody (Santa Cruz Biochemistry Inc.) were used as first antibodies, while Alexa fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 donkey anti-goat IgG (Molecular Prob Inc.) were used as a second antibodies.

#### **Western blot analysis.**

Tissues were homogenized and lysed in a buffer consisting of 150 mM NaCl, 50mM Tris HCl, pH7.5, 0.5% Nonidet 40, 50mM NaF, 1mM Na<sub>3</sub>VO<sub>4</sub>, 1mM PMSF and 1% aprotinin at 4 °C for 30 min. Cell lysates were cleared of cell debris by centrifugation at 14,000g for 30 min.

Twenty  $\mu\text{g}$  of protein were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a PAG mini Daiichi 15/25 gel (Daiichi Pure Chemical Co.). The gel electrophoresis was performed under non-reducing conditions. The proteins were then blotted on a nitrocellulose blotting membrane (Osmonics Inc.). Nitrocellulose membranes were blocked with 5 % BSA, followed by washing with PBS-Tween 20 and incubated with rabbit anti-humanin antibody at 4 °C overnight. After intensive washing, membranes were incubated with horseradish peroxidase-linked goat anti-rabbit IgG, followed by detection with ECL reagents (Biotechs).

#### **Electron Microscope and Colloidal-Gold immunocytochemistry.**

Synovial cells from diffuse type PVNS were gathered in the same manner for light microscopy, and fixed in 3% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C over night. The specimens were postfixed in 1% OsO<sub>4</sub> in 0.1M phosphate buffer (pH 7.4) overnight at 4°C, rinsed three times (10 min each) in 10% saccharose, and stained *en bloc* in 3% aqueous uranyl acetate for 1 hr at room temperature. Samples were then dehydrated in an ascending series of ethanol concentrations, replaced by proplene oxide and embedded in epoxy resin. Ultrathin sections (100 nm) were cut, stained with uranyl acetate and lead citrate, and observed using an electron microscope (Hitachi H-7000).

For electron microscopic immunocytochemistry, cells were fixed in 0.2% glutaraldehyde and 4% paraformaldehyde mixture in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. The samples were embedded into Lowicryl K4M and ultrathin sections (100nm) were used for

incubation with anti-humanin antibody overnight. Incubation with the biotinylated secondary antibody was performed at room temperature for 1 hour, and after washing with PBS and distilled water, incubation with Colloidal-gold streptavidin was performed for 1 hour. After the sections were rinsed and dried, they were stained with uranyl acetate and lead citrate, and electron microscope was performed as described above.

## RESULTS

### **Identification of highly expressed genes in PVNS**

A total of 2956 clones selected by subtraction cloning were further examined by Southern colony hybridization. The sequencing was performed on genes expressed in PVNS at three-times greater frequency than those in RA. Sixty eight of the highly expressed genes were identical to 17 known genes. Two genes were identical to genes encoding two hypothetical proteins. In Table 1, these genes were classified into 7 groups according to their functions and whether they were transcribed in mitochondria. Interestingly, genes encoded in the region of 16S rRNA and 12S rRNA were expressed with high frequencies. Furthermore, we detected various forms of 16S rRNA with poly A tail end, as shown in Fig. 1. There was no 12S rRNA with poly A tail among these genes. The cDNA with polyA tail (16SrRNA•3229: Type 9 in Fig. 1) was identical to the humanin gene.

Northern blot analysis was performed using mRNA of synovial cells from PVNS, RA and OA patients. Humanin genes were strongly expressed in diffuse type PVNS, but were barely

detectable in nodular type PVNS, RA, or OA (Fig. 2). However, other genes encoded by mitochondria were not increased as assessed by semiquantitative RT-PCR, suggesting that ribosomal genes were selectively expressed in mitochondrial genes in PVNS (Fig. 3). This is the first report of the expression of humanin gene in synovial cells.

### **Expression of the humanin peptide in PVNS**

Next, the expression of humanin peptide was identified using synovial cell lysates from diffuse type PVNS and anti-humanin polyclonal anti-body (Fig. 4). Immunohistochemical analysis showed that most of the positive cells were distributed in the deep layer (Fig. 5). This positive staining was thoroughly suppressed by blocking the primary antibody with synthesized antigen peptide (data not shown).

Although it has been suggested that the humanin peptide is expressed by cells in the deep layer of PVNS, little is known about the intracellular localization of this peptide. In further examinations, we detected intracellular humanin peptide in synovial cells from diffuse type PVNS. The humanin peptide was stained with red color which localized in the cytoplasm of the synovial cells (Fig.6-b) but not in the nucleus. Mitochondria was stained with green color using anti-heat shock protein 60, which is mitochondrial specific chaperonin (Fig. 6-c). Double staining with anti-humanin antibody and anti-heat shock protein 60 (yellow color) demonstrated that humanin was expressed mainly in mitochondria (Fig.6-d).