We examined the in-vitro effects of drugs by comparing PATI values obtained from drug-treated WB at 60 min after blood collection. Aspirin and cilostazol inhibited WB aggregation in a concentration-dependent manner but in a non-linear fashion, while ramatroban inhibited linearly with increasing concentration. The difference in the increased pattern of the PATI values may be due to differences in pharmacological mechanisms between the three drugs. In our unpublished data, sarpogrelate hydrochloride, a 5-HT_2-serotonergic receptor antagonist, inhibited WB aggregation induced by simultaneous use of low concentration of collagen and serotonin linearly with increasing concentration from 0.1 to 100 μmol/l, showing a 200–500% increase of PATI values. It is of interest that enzyme inhibitors such as aspirin and cilostazol showed non-linear inhibition on WB aggregation, while receptor antagonists such as ramatroban and sarpogrelate suppressed in a linear fashion. The increase of PATI in PRP aggregation induced by ADP (1, 2, 4 and 8 μmol/l) showed a similar pattern to WB aggregation (Fig. 7). However, increases of PATI of 100 μmol/l aspirin and 10 μmol/l cilostazol on WB aggregation in response to ADP showed 292 and 272% increases, respectively, and were lower than that of 1 μmol/l ramatroban (388%) (Fig. 7a), and are different from the results of PRP aggregation induced by 5 μmol/l ADP (Fig. 3a).

We obtained confocal images that latticework openings on the microsieve after WB aggregation induced by ADP were filled with aggregates. Confocal fluorescence laser microscopy confirmed that the aggregates contained platelets and leukocytes. Thus, WB aggregometry using the screen filtration pressure method most probably reflects not only platelet–platelet aggregates, but also platelet–leukocyte conjugates.

In conclusion, we have more clearly clarified the difference in anti-platelet effects of drugs by investigating both PRP aggregation and release reaction in response to various agonists. It was demonstrated that the measurement of WB aggregation is also useful to evaluate the effects of drugs on platelet aggregation.

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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 cytoplasma 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^+ 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^{TM} cDNA Subtraction Kit (Clontech). Equal amounts of double-stranded cDNAs from two patients with PVNS (diffuse type / lane 1, nodular type / lane2 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 [^{32}P]-dCTP by random priming (Stratagene). Membranes were hybridized in aqueous solution (5 x SCC, 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°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°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 minutes for 25 cycles). The cDNAs were purified and sequenced using the ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kit with Template Suppression Reagent (ABI PRISM^{TM}). DNA sequences were analyzed using DNASIS software and compared to sequences in GeneBank (National Center for Biotechnology Information, Bethesda, MD).

Northern hybridization. Poly A^+ 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^{TM}-N+ nylon transfer membrane (Amersham). Purified human cDNA (40 ng) was labeled with [^{32}P-dCTP] by *random priming* and applied to the membrane forhybridization in aqueous solution (5 x SCC, Denhardt's solution, 0.1% SDS, 10mg salmon sperm DNA, 50% formamide) overnightat 42°C. After washing at 42°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°C.

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

Total RNA (2.5 μg) from 5 patients with PVNS, 3 with RA and 2 with OA were used for cDNA

synthesis with oligo$(dT)_{12-18}$ 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 $MgCl_2$ (Takara Biomedical), 0.4 μM forward and reverse primers, and 2.5U Taq polymerase (Takara). All amplimers were amplified simultaneously with GAPDH as internal standard. The respective primer pairs were for cytochrome c (forward; 5'-GCATAAACAACATAAGCTTCTGA-3',reverse;5'-CAGCAGATCATTTCATATTGCTT-3'), for ATPase (forward; 5'-TCTCATCAACAACCGACTAATCA-3', reverse; 5'-GATAAGTGTAGAGGGAAGGTTAA-3'), for NADH dehydrogenase (forward; 5'-TTTACTCAATCCTCTGATCAGGG-3', reverse; 5'-CGAATTCATAAGAACAGGGAGGT-3'), and for cytochrome b (forward; 5'-AATTACAAACTTACTATCCGCCA-3', reverse; 5'-TGGGCGAAATATTATGCTTTGTT-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_3VO_4, 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 μg 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_4 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 colar 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).

Electron microscopic observation of synovial cells from diffuse type PVNS revealed that most of the iron deposits were included within the siderosome as described previously.(16,17) However, some electron dense iron deposits were observed within mitochondria (Fig.7-a). Interestingly, mitochondrial membrane debris with electron dense iron deposits were observed within the siderosome which was characterized as an autophagosome (Fig.7-a). On the other hand, some normal mitochondria were scattered throughout the cytoplasm (Fig7-b). Electron dense iron deposits within the siderosome were observed by electron microscopic immunohistochemistry (Fig. 8). In some siderosomes, particles of colloidal-gold were precipitated to the debris adjacent to electron dense iron (Fig. 8-a). These results suggest that humanin exists in mitochondria not only in the cytoplasm but also in the siderosome after being phagocytosed.

DISCUSSION

Genes with enhanced expression in synovial cells from PVNS were grouped according to their functions and the transcription in mitochondria as listed in Table 1. It is likely that many of the listed genes may be involved in the pathogenesis of PVNS according to their characterized functions. Interestingly, genes encoded in the regions of 16S rRNA and 12S rRNA were expressed with high frequencies. Previous reports pointed out the presence of polyadenylated transcripts of the 16S rRNA gene that were different from the 16S rRNA. (24,25,26) These poly A sequences are considered to be due to active metabolism of mitochondria in cancer cells, since the increased expression of the 16S rRNA genes was found

only in malignancies. (19,27) These facts suggest that the genes encoded in the region of rRNA from PVNS reflect the neoplastic nature of this disease. In fact, for PVNS, especially the diffuse type, the neoplastic hypothesis is supported by the demonstration of aneuploid DNA content and the existence of cytogenetic aberration, as well as the capacity of these lesions for autonomous growth and the potential for recurrence. (13,14)

It is intriguing to examine whether these mitochondrial genes for 16S rRNA are virtually translated and act as functional peptides. In this regard, humanin is a polypeptide described as a rescue factor abolishing neural cell apoptosis. This peptide protects neural cells of the F11 line from death induced by the expression of mutated genes, causing early-onset familiar Alzheimer's disease.(28) Additionally, it was reported that humanin protects CN-procaspase-3 from amyloid precursor protein-induced cleavage, thereby preventing apoptosis.(29,30,31) More recently, Guo et al. (20) also described the anti-apoptotic mechanism of this peptide through interference with Bax activation. In this study, we proved that the humanin peptide, encoded in the mitochondrial genome, was selectively expressed in the mitochondria and within the siderosome in the diffuse type PVNS synovial cells. It is well established that damaged and functionally disabled mitochondria may be autophagocytosed by lysosomes to prevent continuous oxidative damage, as shown in the degenerating mitochondria within the siderosome in our electron microscopic study. (32,33) This evidence suggests that humanin is translated in mitochondria, causing survival of this organelle under the condition of excessive iron deposition.

In fact, extreme iron deposition is one of the most characteristic pathological features in PVNS. (2,7,8) This deposit is derived from the breakdown of erythrocytes that are phagocytosed after repeated bleeding into the joint space. (16,34,35) Under the condition of iron excess, some of the iron is shunted into hemosiderin and stored in the cytoplasm. (36) It is well described that reactive oxygen species are generated by excessive iron-induced cell apoptosis, which is one important mechanism implicated in the mitochondrial death pathway. (36,37,38,39,40) This mechanism may involve the capacity of excessive iron deposits to stimulate lipid peroxidation, thereby disrupting lysosomal membranes and releasing tissue destructive hydrolytic enzymes.(41,42) In regard to PVNS, as shown in our subtraction cloning, the iron deposits are known to be associated with large quantities of ferritin. Nevertheless, homogeneous synovial cells with small, rounded siderosomes in the deep layer of synovium, which present predominantly in diffuse type PVNS, were reported to have minimum tissue damage adjacent to the iron deposits.(43) Morris et al. (43) reported that electron dense iron deposits were associated with mitochondrial destruction in haemophilic synovitis but much less in PVNS. Several explanations were described for this lack of mitochondrial damage in previous reports, such as transitional function during inflammation, or the failure of the apoferritin response.(31,43) However, there were no facts to explain this pathology.

The alternative intriguing explanation about this pathogenesis of PVNS is that a mitochondrial abnormality exists primarily in PVNS independent of the precipitation of hemosiderin. In that case, the overload of iron deposits in the cytoplasm and mitochondria could induce free radicals.

However, abnormal mitochondria would be responsible for supplying a key reactant, humanin, to prevent oxidative damage until they are autophagocytosed within siderosome, resulting in cell survival. In accordance with this view, analysis of isolated cells has enabled us to describe here for the first time the feature of hemosiderin-containing mitochondria, which was autophagocytosed and degenerating within the siderosome, in addition to many mitochondria without hemosiderin scattered around the cytoplasm.

Taken together, our findings lead us to a simple interpretation that the possible function of humanin located within the mitochondria in PVNS synovial cells may be to serve as a rescue factor from excessive iron damage and consequent organelle breakdown in the cytoplasm and cell death. However, Hashimoto et al. (19,23) have shown that cell death is only supported by the secreted humanin peptide, and the function of the peptide located intracellularly is still unclear. Although future studies are required to investigate the function of humanin within the cytoplasm, our data suggest that humanin is involved in the iron depositing pathology of PVNS.

In conclusion, our results suggest that the humanin peptide is highly expressed in the synovial cells from diffuse type PVNS and may be involved in the pathology of PVNS.

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