Increased expression of humanin peptide in diffuse-type pigmented villonodular synovitis: implication of its mitochondrial abnormality


OBJECTIVES: To define the pathogenesis of pigmented villonodular synovitis (PVNS), by searching for highly expressed genes in primary synovial cells from patients with PVNS.

METHODS: A combination of subtraction cloning and Southern colony hybridisation was used to detect highly expressed genes in PVNS in comparison with rheumatoid synovial cells. Northern hybridisation was performed to confirm the differential expression of the humanin gene in PVNS. Expression of the humanin peptide was analysed by western blotting and immunohistochemistry. Electron microscopic immunohistochemistry was performed to investigate the distribution of this peptide within the cell.

RESULTS: 68 highly expressed genes were identified in PVNS. Humanin genes were strongly expressed in diffuse-type PVNS, but were barely detected in nodular-type PVNS, rheumatoid arthritis, or osteoarthritis. 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 disclosed immunolocalisation of this peptide, predominantly around dense iron deposits within the siderosome.

CONCLUSIONS: Increased expression of the humanin peptide in mitochondria and siderosomes is characteristic of synovial cells from diffuse-type PVNS. Humanin is an anti-apoptotic peptide which is encoded in the mitochondrial genome. Present findings suggest that mitochondrial dysfunction may be the principal factor in pathogenesis of diffuse-type PVNS and that humanin peptide may play a part in the neoplastic process in this form of PVNS.

Pigmented villonodular synovitis (PVNS) is classified as an uncommon idiopathic proliferative synovial process. It can exist in a localised form within a joint, but more commonly occurs as a diffuse form where the entire synovium of a joint is affected. The exact aetiology of PVNS is still unknown. Previous experimental and epidemiological studies have suggested that PVNS is a reactive process involving a chronic inflammatory response. However, recent studies show the capacity for these lesions for autonomous growth and the potential for recurrence have suggested involvement of a neoplastic process. 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.

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 and Gladially et al reported that the siderosome fuses with mitochondria in deep synovial cells from patients with PVNS. Moreover, abundant mitochondria throughout the cytoplasm were observed in dispersed stromal cells containing electron dense inclusions and in giant cells.

In this study, we searched for highly expressed genes in primary synovial cells from patients with PVNS compared with those from patients with rheumatoid arthritis (RA). We considered that a 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 was highly expressed in PVNS. Among these genes, humanin has been reported to act as an oncogene or as an anti-apoptotic factor against Bax (Bcl2 associated X protein), which is an apoptosis-inducing protein. 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 with other arthritides 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 six patients with PVNS, three with RA, and three with osteoarthritis (OA). These lesions were subtyped into two types (diffuse or nodular) according to locations (intra-articular versus extra-articular) and pathological growth patterns, which reflected clinical characteristics and biological behaviour.

Abbreviations: BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hsp, heat shock protein; IMDM, Iscove modified Dulbecco’s medium; OA, osteoarthritis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PVNS, pigmented villonodular synovitis; RA, rheumatoid arthritis; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SSC, saline sodium citrate
The patients with RA met the criteria of the 1987 American College of Rheumatology. The tissue was cultured in Iscove modified Dulbecco’s medium (IMDM) with collagenase V (1 mg/ml medium) for 40 minutes and cells were harvested through mesh and gathered by centrifugation. Total cellular RNA was extracted using acid-guanidinium-phenol-chloroform (AGPC) methods. 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 microgram of total RNA sample was used to synthesise full length, double stranded cDNA using a SMART polymerase chain reaction (PCR) cDNA synthesis kit (Clontech). Subtraction cloning was performed with 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 three patients with RA were used as driver cDNAs. PCR using CD163 primers was performed to estimate the efficiency of subtraction, and the expected decrease in CD163 in the subtracted sample was observed (data not shown).

**Southern colony hybridisation**

Subtracted cDNAs were ligated to TOPO vector (Invitrogen) and transformed into DH10B cells (Invitrogen) by electroporation. After blue-white selection with X-gal containing Luria-Bertani (LB) plates, white colonies were cultured overnight with 150 μl LB medium in sterile 1.5 ml tubes, white colonies were cultured overnight with 150 μl LB medium in sterile 1.5 ml tubes and centrifuged for 2 minutes at 12 000 g, and the pellet was resuspended in 10 μl LB medium. The medium was mixed completely and 2 μl was dotted onto a nylon membrane for Southern hybridisation. SMART double stranded cDNA was labelled with [32P]dCTP by random priming (Stratagene). Membranes were hybridised in aqueous solution (5 x saline sodium citrate (SSC), Denhardt’s solution, 0.1% sodium dodecyl sulphate (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. Measurement of cDNA was performed by scanning with a BAS 1000 Densitometer (Fuji film), and normalisation against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA hybridised subsequently on the same blots.

**DNA sequencing**

Sixty eight cDNAs from differentially expressed clones were amplified with M13 reverse (5'-CAGGAAAAACGCTATGAC-3') 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 dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Purified human cDNA (40 ng) was labelled with [32P]dCTP by random priming (Stratagene). Membranes were hybridised in aqueous solution (5 x SSC, Denhardt’s solution, 0.1% SDS, 10 mg salmon sperm DNA, 50% formamide) overnight at 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.

**Northern hybridisation**

Poly A+ RNA (168 ng) samples of the synovium from five patients with PVNS, three with RA, and three with OA patients were loaded and fractionated through 1.0% agarose gels and transferred to Hybond-N+ nylon transfer membrane (Amersham). Purified human cDNA (40 ng) was labelled with [32P]dCTP by random priming and applied to the membrane for hybridisation in aqueous solution (5 x SSC, Denhardt’s solution, 0.1% SDS, 10 mg salmon sperm DNA, 50% formamide) overnight at 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 five patients with PVNS, three with RA, and two with OA was used for cDNA synthesis with oligo(dT)25 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 μmol/l of each dNTP, 1 x PCR buffer including 1.5 mM MgCl2 (Takara Biomedical), 0.4 μM forward and reverse primers, and 2.5 U Taq polymerase (Takara). All amplifiers were amplified simultaneously with GAPDH as internal standard. The respective primer pairs were for cytochrome c (forward: 5'-GCCATCAAAAATGAGGTGAGT-3', reverse: 5’-CAGGAGATCCTGGAGCTG-3'), for ATPase (forward: 5’-TCTCTACACAAACCAGACTAAT-3', reverse: 5’-GATAATGCTAGGGAAGGTAAA-3'), for NADH dehydrogenase (forward: 5’-TTACTCAATCTCTGCATGGG-3', reverse: 5’-CAATTCAAGAAACGAGGAG-3'), and for cytochrome b (forward: 5’-AATTAAACACTTATCCATG-3', reverse: 5’-TGGGGCAAATATTGCTTCTGTT-3'). The reaction mixtures were incubated for 3 minutes at 94°C, followed by 32 cycles of denaturation for 1 minute at 94°C, annealing for 1.5 minutes at 52°C, and extension for 1 minute at 72°C.

**Cell and tissue processing for light microscopy and immunohistochemistry**

To isolate synovial cells, the deep layers of synovium from diffuse-type PVNS were cultured in IMDM with collagenase...
V (1 mg/ml medium) for 20 minutes and cells were harvested through mesh and gathered by centrifugation. These synovial cells were cultured in IMDM with 10% fetal bovine saline for 4 hours and fixed with 10% buffered formaldehyde at room temperature for 10 minutes, then rinsed with phosphate buffered saline (PBS). Formalin fixed tissue sections were also used for immunostaining. A rabbit polyclonal anti-humanin antibody was synthesised and purified on an affinity column and dissolved in PBS (0.9% NaCl, 0.02 M phosphate buffer, pH 7.0). The IgG concentration was analysed using a protein assay kit (Bio-Rad). Immunostaining was performed as previously described.22 23 Briefly, cells and sections were fixed with 4% formaldehyde in PBS. After rinsing 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% bovine serum albumin (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 antirabbit IgG (Molecular Probes Inc) was applied as a secondary antibody for 60 minutes. Immunofluorescence was detected with a CSU-10 confocal laser scanning unit (Yokogawa Electric Co), coupled to an IX90 inverted microscope with UPlanAPOX20 objective lens (Olympus Potalic Co), and CS810–01 colour chilled 3CCD camera (Hamamatsu Photonics, KK). For double staining, anti-humanin antibody and anti-heat shock protein (hsp) 60 antibody (Santa Cruz Biochemistry Inc) were used as first antibodies, while Alexa Fluor 568 goat antirabbit IgG and Alexa Fluor 488 donkey antigoat IgG (Molecular Probes Inc) were used as a second antibodies.

Western blot analysis

Tissues were homogenised and lysed in a buffer consisting of 150 mM NaCl, 50 mM Tris HCl, pH 7.5, 0.5% Nonidet 40, 50 mM NaF, 1 mM Na3VO4, 1 mM phenylmethylsulphonyl fluoride, and 1% aprotinin at 4 °C for 30 minutes. Cell lysates were cleared of cell debris by centrifugation at 14 000 g for 30 minutes. Protein (20 μg) was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on

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Figure 1 The sequences encoded within the 16S rRNA region with poly A tail. The cDNA fragments were aligned with the 16S rRNA region of the mitochondrial gene and the correlating humanin mRNA sequence. Southern colony hybridisations repeated these sequences in a total of three rounds independently. Oblique bars show the digestion sites by Rsa I and upward diagonal bar shows the region of humanin coding sequences. Although there are nine types of sequences with poly A tail within this region, only the type 9 sequence was identical to the previously reported mRNA encoding humanin peptide.

Figure 2 Northern blot analysis of mRNAs expressed by synovial cells from patients with PVNS, RA, and OA. Total RNA (168 ng) was subjected to electrophoresis in a 1.0% agarose gel containing formaldehyde, transferred to a nylon membrane, and probed with [32P]dCTP labelled cDNA (type 9; fig 1). Another cDNA (type 3) encoded in the 16S rRNA region was also used in northern blotting and the expression level and size were same as those using type 9 cDNA (data not shown). Humanin genes were strongly expressed in diffuse-type PVNS, but barely detected in nodular-type PVNS, RA, or OA. The size of the expressed major message was ~1.6 kb and the other messages were ~1 kb, which corresponds to the results of a previous report by Hashimoto et al.28
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 onto a nitrocellu-
lose 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, mem-
branes were incubated with horseradish peroxidase linked
horseradish peroxidase linked
goat antirabbit IgG, followed by detection with enhanced
chemiluminescence reagents (Biotech).

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 0.1 M phosphate buffer (pH 7.4) at 4˚C
overnight. The specimens were postfixed in 1% OsO 4 in
0.1 M phosphate buffer (pH 7.4) overnight at 4 ˚C, rinsed
three times (10 minutes each) in 10% saccharose, and
stained all together in 3% aqueous uranyl acetate for 1 hour
at room temperature. Samples were then dehydrated in an
ascending series of ethanol concentrations, replaced by
propylene 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 (100 nm) 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. The sections were rinsed and
dried, then stained with uranyl acetate and lead citrate, and
electron microscopy 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 hybridisation. 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. Table 1 classifies these genes into seven 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. No 12S
rRNA with poly A tail was found among these genes. The
cDNA with poly A tail (16S rRNA N3229: type 9 in fig 1) was
identical to the humanin gene.

Northern blot analysis was performed using mRNA of
synovial cells from patients with PVNS, RA, and OA. 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). As far as we know, this is the first report of the expression of the 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 antibody (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 synthesised 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 localisation of this peptide. In further examinations, we detected intracellular humanin peptide in synovial cells from diffuse-type PVNS. The humanin peptide was stained with a red colour, which localised in the cytoplasm of the synovial cells (fig 6B) but not in the nucleus. Mitochondria were stained with a green colour using anti-hsp60, which is mitochondrial-specific chaperonin (fig 6C). Double staining with anti-humanin antibody and anti-hsp60 (yellow colour) demonstrated that humanin was expressed mainly in the mitochondria (fig 6D).

Electron microscopic observation of synovial cells from diffuse-type PVNS showed that most of the iron deposits were included within the siderosome as described previously. However, some electron dense iron deposits were

Figure 5  Synovial tissue from diffuse-type PVNS was fixed with 4% formaldehyde in PBS. The specimens were stained with anti-humanin antibody, followed by Alexa 488 goat antirabbit IgG, and photographed with a fluorescent microscope (×40). (A) Most positive cells (green) were distributed in a deep layer with haemosiderin deposit. (C) Negative control of the continuous section. (B, D) Backgrounds for (A) or (C), respectively.

Figure 6  Relationship between humanin peptide expression and mitochondria. Isolated synovial cells containing haemosiderin were double stained with anti-humanin antibody and anti hsp60 antibody as first antibodies, followed by goat antirabbit IgG and donkey antigoat IgG as second antibodies (×400). (A) Haemosiderin was deposited unequally throughout the cytoplasm. (B) Single anti-humanin antibody staining (red). (C) Single anti-hsp60 antibody staining (mitochondrial staining; green). (D) Humanin was dominantly distributed in the mitochondria around the siderosome (yellow).
observed within the mitochondria (fig 7A). Interestingly, mitochondrial membrane debris with electron dense deposits was observed within the siderosome as an autophagosome (left arrow). (B) Some of the normal mitochondria (arrows) also were scattered throughout the cytoplasm. (Magnification x 19 000.)

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 play a part in the pathogenesis of PVNS according to their characterised 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 differed from the 16S rRNA.24–26 These poly A sequences are considered to be due to active metabolism of mitochondria in cancer cells, because the increased expression of the 16S rRNA genes was found only in malignancies.24–27 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.29–31 More recently, Guo et al also described the anti-apoptotic mechanism of this peptide through interference with Bax activation.32 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, leading to 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. This deposit is derived from the breakdown of erythrocytes that are phagocytosed after repeated bleeding into the joint space. Under the condition of iron excess, some of the iron is shunted into haemosiderin and stored in the cytoplasm. 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. This mechanism may involve the capacity of excessive iron deposits to stimulate lipid peroxidation, thereby disrupting lysosomal membranes and releasing tissue destructive hydrolytic enzymes. In regard to PVNS, as of excessive iron deposits to stimulate lipid peroxidation, pathology.

peptide is highly expressed in the synovial cells from diffuse-PVNS. In conclusion, our results suggest that the humanin that humanin is involved in the iron depositing pathology of PVNS. Although future studies are required to investigate the function of the peptide located intracellularly is still unclear. 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. Morris et al reported that electron dense iron deposits were associated with mitochondrial destruction in haemophilic synovitis but much less so in PVNS. Several explanations were suggested for this lack of mitochondrial damage in previous reports, such as transitional function during infarlation, or the failure of the apopferitin response. 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 which is independent of the precipitation of haemosiderin. 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 the 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 mitochondria containing haemosiderin, which were autophagocytosed and degenerating within the siderosome, in addition to many mitochondria without haemosiderin 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 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 play a part in the pathology of PVNS.

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