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Apoptosis resistance in pigmented villonodular synovitis

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Summary. Objective: Pigmented villonodular synovitis (PVNS) is a proliferative lesion originating from synovial tissue with a locally aggressive behaviour. We analysed the pathogenetic role of apoptosis resistance for sustained cell proliferation in PVNS. Methods: The expression of bcl-2, p53 and Ki-67 was examined in 80 cases of PVNS using immunohistochemistry. In 43 of these cases, DNA content and distribution of cell-cycle phases were investigated by flow cytometry. Additionally, 10 cases of PVNS were analysed by multi-parametric flow cytometry for expression of p53, caspase3, and bcl-2 and by TUNEL to detect DNA fragmentation. Results: No apoptotic cell fractions were detected in any investigated cases. Expression of bcl-2 was found in 84% of cases (up to 6.5% of cells) and was significantly associated with DNA-fragmentation observed by TUNEL (p=0.037). Orthologous p53 expression was observed in 37% of cases. The level of p53 expression correlated with the proliferative activity and the expression of both caspase3 (p=0.017) and bcl-2 (p=0.0013). (No statistically significant correlations between expression of bcl-2, p53, caspase3, DNA fragmentation or proliferative index and age, sex of patients, disease recurrence, growth pattern or size of lesion were found). Conclusion: Apoptosis resistance is a critical event in the progression of PVNS and may contribute to the survival of the proliferating synovial cells in PVNS and to the permanent slow progression of these lesions.

Key words: bcl-2, Apoptosis resistance, PVNS

Introduction

Pigmented villonodular synovitis (PVNS) is an aggressive proliferative lesion originating from the synovial membranes of tendons and joints. Manifestation of the disease is seen most frequently as a monoarticular lesion in knee and hip joints. However, oligo- and polyarticular lesions involving other joints have been described (Weidner et al., 1986; Clerc et al., 1999).

Histological features of PVNS include proliferation of synovial cells with a scant, non- foamy cytoplasm and small nuclei, scattered osteoclast-like giant cells, an inflammatory infiltrate with focal accumulation of lipid-loaded macrophages and deposits of iron pigment (Yoshida et al., 2003). A nodular as well as a diffuse growth pattern can be distinguished (Fassbender, 2002).

The nature of the disease entity has been a matter of controversy with both inflammatory and neoplastic processes being discussed (Aigner et al., 1998; Clerc et al., 1999; Perka et al., 2000). However, most authors regard this lesion as a benign neoplasm with a relatively high recurrence rate (Mertens et al., 1993; Ohimi et al., 1996; Clerc et al., 1999). Furthermore, singular recurrent cases of PVNS with histological features of malignancy and pulmonary or nodal metastases have been reported (Layfield et al., 2000).

Apart from existent uncertainties concerning the biological nature of PVNS, clinical and morphological characteristics of this disease indicate an ongoing proliferative process. However, the mechanisms triggering proliferation are still not completely understood. In recent studies, chromosomal aberrations (Mertens et al., 1993; Ohimi et al., 1996), aneuploidy in PVNS and alterations in the expression pattern of cell-cycle-related gene products (Weckauf et al., 2004) have been demonstrated. The pathogenic role of apoptosis resistance in the progression of PVNS is still not clear.

Apoptosis is considered a rescue mechanism that controls the integrity of the cell by erasing aberrant clones (Gianetti et al., 2004). Cell death by apoptosis mediates several important physiological and pathological processes. Dysregulation of the programmed cell death constitutes a key factor responsible for unregulated cell proliferation and probably plays a major role in many types of diseases, including neoplasias (Rudin and Thompson, 1997). One of the best-studied genes regulating programmed cell death is bcl-2, a member of a growing family of genes that participate in the control of apoptosis. The bcl-2 protein blocks a distal step in a pathway for programmed

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cell death (Wan and Reed, 1998). The family of caspase enzymes, currently including 14 members, is subdivided by the prodomain length, specific substrate and phylogenetic analysis, and plays a crucial role in the apoptotic cascade. A growing number of investigations report that p53 induces apoptosis. However, p53 expression as such is not sufficient for cell death (Yonish-Rouach et al., 1991; Smith and Firnace, 1995; Martin et al., 1996).

In this study we investigated different aspects of apoptosis resistance in localized and diffuse PVNS to examine whether the characteristic proliferative behavior of PVNS could be influenced by apoptosis resistance. For this purpose we analyzed tissue samples from patients with different forms of PVNS by using multi-parametric flow cytometry with combined determination of DNA-content and DNA-fragmentation by TUNEL in addition to analysis of expression of p53, bcl-2, caspase3 and Ki-67 by using immunoflow-cytometry and immunohistochemical stainings performed on tissue microarrays.

Materials and methods

Clinical data

The following clinical data were collected using a questionnaire: age and sex of patients, affected joints, number of affected joints, clinical diagnosis, duration of the symptoms, clinical behavior, recurrence of disease and size of tumors.

Tissue

Material retrieved by synovialectomy taken from 80 patients with localized (23) and diffuse (57) PVNS, collected between 1989 and 2003 were selected from the tissue bank of the Centre of Rheuma-Pathology, WHO-collaborating Center, Mainz, Germany and the Institute of Pathology, University of Heidelberg, Germany. Diagnosis of PVNS was established by conventional histological criteria (Aigner et al., 1998; Fassbender, 2002). All samples were fixed in 4% formaldehyde and paraffin- embedded via routine procedures

Tissue microarrays

Sections were made from each paraffin block and stained with hematoxylin and eosin (HE). As recommended in previous studies, representative regions of PVNS were selected. From each of these regions two tissue cylinders measuring 1.5 mm in diameter were obtained from the corresponding areas of the paraffin block. Normal synovial tissue (N=10) was used as control. The cylinders were arrayed into a recipient paraffin block using a tissue chip microarrayer (Beecher Instruments, Silver Springs, USA). Subsequent 3-µm sections were cut from the recipient block and mounted on sialinised glass slides to support adhesion of the tissue samples.

Immunohistochemistry

Sections of the tissue array were deparaffinized and subjected to heat-induced antigen retrieval using citrate buffer (Dako, Hamburg, Germany) in a microwave oven. Immunostaining was performed on an automated staining system (Techmate Horizon, Dako, Hamburg, Germany) using the immunoperoxidase method and AEC (amino-ethyl-carbazole) as chromogen. Dilution of primary antibodies was 1:50 for bcl-2 (Clone 124, Dako), 1:100 for p53 (DO7, Dako) and 1:200 for Ki-67 (MIB1, Dianova, Hamburg, Germany).

Quantitative evaluation

Quantitative evaluation was performed independently by two pathologists using a semiquantitative score ranging from 0-3: score 0, no staining, score; 1, positive cytoplasmatic staining in less than 10% of proliferating cells; score 2, positive cytoplasmatic staining in 10-50% of cells; score 3, positive cytoplasmic stain in more than 50% of proliferating cells. If the scores between the two samples taken from each case differed the sample with the highest score was used for evaluation.

Proliferating synovial mononuclear cells, foamy cells, osteoclast-like giant cells and inflammatory infiltrations were analyzed separately.

Statistical analysis of data

Statistical analysis was performed by using the R (1.7.1) software package. Count data were analyzed by using Fisher's Exact test; for continuous data the Wilcoxon rank test was used. Statistical correlations for comparative analysis of expression patterns of the investigated parameters were performed using Spearman's Rank Correlation test.

DNA flow cytometry (DNA-FCM)

Preparation of a single cell suspension from paraffin-embedded material was made according to the Hedley technique (Hedley et al., 1983) with modifications (Sergi et al., 1999).

From blocks with paraffin embedded tissue non-tumorous tissue was removed using a fine glass needle, a stereomicroscope with low magnification and a conventionally stained section as reference.

From the remaining tumorous tissue, 50-µm tissue sections were cut, dewaxed using two changes of xylene, rehydrated and washed twice in distilled water. The sections were digested in 0.9% NaCl (pH 1.5) containing 0.5% pepsin (Serva, Heidelberg, Germany) for 30 min at 37 °C with intermittent vortex mixing. The single cell suspensions (absolute cell number: 30,000-110,000) were stained in 1µg/ml 4',6-diamidino-2-phenylindole

(DAPI) (Fa. Parec, Münster, Germany) in TRIS-buffer (pH 7.8) according to the method of Otto with modifications (Otto, 1994; Ehemann et al., 1999).

A preparation of normal human lymphocytes was used to calibrate the G0/G1 diploid peak. The DNA content was measured in a PAS II flow cytometer (Partec, Münster, Germany) with a 100 W mercury vapor lamp. The following filters were used: KG 1, BG 38 and UG 1 for excitation; TK 420 as dichroic mirror; and GG 435 as barrier filter. The fractions of cells in cell-cycle phases G0/G1, S and G2/M phases were calculated by a computerised mathematical model as well as the DNA-Index for diploid and aneuploid stem lines (Multicycle, Phoenix Flow System, San Diego, California, USA) (Rabinovich, 1993; Kallioniemi et al., 1994).

DNA fragmentation was detected by TUNEL. This is a method based on the terminal deoxynucleotidyl transferase mediated d-UTP-biotin nick end labelling (TUNEL) to detect DNA strang breaks, which represent very early events in apoptosis.

After separation of single cells, the cell population was first fixed in 1.0% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4, followed by three washes in PBS and fixation in ice-cold 70% (v/v) ethanol for 30 min at -20 °C. Then, cell suspensions were centrifuged at 800 rpm for 10 min and washed twice in phosphate-buffered saline at pH 7.4. DNA-fragmentation was detected with a TUNEL kit (Roche, Mannheim, FRG) according to the manufacturers instructions at 37 °C for 60 min in the dark. Finally, fluoresceine TUNEL positive cells as well as unlabelled cells were counter-stained with DAPI (25µg/ml) for DNA quantification (Ehemann et al., 2003). Green fluorescence (FITC) was detected on channel one in the logarithmic mode and, red fluorescence Phycoerytrine (PE) in channel 2 in the logarithmic mode, while UV fluorescence (DAPI) was recorded in the linear mode on channel six. P53-PE (Pharmingen, Heidelberg, FRG), Bcl-2-FITC (Pharmingen, Heidelberg FRG) and, active caspase-3-PE (Pharmingen, Heidelberg FRG) assays were done in same way with incubation time of 30 min in the dark at room temperature, followed by counter staining with DAPI (25 µg/ml). All the antibodies, p53, bcl-2 and active caspase-3, were measured against the cell cycle. For each measurement 10,000 – 50,000 cells were analyzed. The multi-parameter analyses were measured in a Galaxy pro-flow cytometer (Partec, Münster FRG) equipped with a 488nm Argon laser to detect the FITC- and PE-marked antibodies and a 100 W mercury vapor lamp to simultaneously detect the DNA content and cell-cycle phase.

For multi-parametric calculations and analyses we used the Flow-Max-Program (Partec, Münster FRG). Cut-off for negative and positive cells resulted from FITC-fluorescence isotype control (Pharmingen, Heidelberg FRG) and PE –fluorescence isotype control (Pharmingen, Heidelberg FRG) measurements for each sample.

Table 1. Bcl-2 expression in mononucleated synovial cells in PVNS by analysis of tissue arrays.

POSITIVE STAINING	LOCALIZED PVNS	DIFFUSE PVNS
< 10% (Score 1)	65.2% (15/23)	68.2% (39/57)
10-50% (Score 2)	17.3% (4/23)	15.9% (9/57)
Total	82.7% (19/23)	84.1% (48/57)

Results

Analysis of clinical data

20 male and 40 female patients (male/female ratio: 1/2) were treated for primary disease (diffuse PVNS: n=43; localized PVNS: n= 17). 20 patients were operated for recurrent disease (diffuse PVNS: n=15; localized PVNS: n=5; male: n=7; female: n=13). For 5 patients, both primary manifestation and recurrence were analyzed. In the remaining 15 cases of relapsing PVNS, the primary lesion was not available for analysis. 76 patients presented with monoarticular disease; in 4 cases (2 diffuse and 2 localized lesions) more than one joint was involved. The most commonly affected joint was the knee (71 cases, 91%); PVNS of the hip (3 cases, 4%) or the radioulnar joint (4 cases, 6%) was diagnosed less frequently.

The age at diagnosis ranged from 14 to 74 years and the average diameter of the lesions was 5 cm in diffuse PVNS (ranging from 0.5 to 14 cm) and 2.8 cm in localized lesions (ranging from 1 to 12 cm).

Immunohistochemistry

We investigated expression of bcl-2, p53 and Ki-67 separately in different cell populations involved in PVNS, such as mononucleated synovial cells, osteoclast-like giant cells, lipid-loaded macrophages, pigment-storing cells and mononuclear inflammatory cells.

In all samples of localized and diffuse PVNS, the bcl-2, p53 and Ki-67 stainings were negative in lipid-loaded macrophages and in pigment-storing cells (Fig. 1b-d).

Some giant cells showed a weak cytoplasmic positivity for bcl-2.

Mononucleated synovial cells stained positive for bcl-2 in 83.75% of cases (67/80).

Expression of bcl-2 is summarized in Table1.

Comparative analysis of bcl-2 expression in primary and recurrent PVNS did not reveal any statistically significant differences (p=0.905). No statistically significant correlations between bcl-2 expression and age (p=0.7575) or sex of patients (p=1.0000) or between bcl-2 expression and size of lesions (p=0.6936) were found.

P53 expression was detected in both forms of PVNS (diffuse: 34%, localized: 39.1%). There was no

significant difference (p=0.794).

An expression of p53-protein was found in 80% (16/20) of recurrent PVNS and in 59.2% (32/54) of the primarily diagnosed villonodular synovitis (p=0.0035). Overexpression of p53 in more than 10% of the tumor cells was found in 40% (8/20) of recurrent PVNS and in 29.6% (16/54) of primary lesions. There was no statistical significance (p= 0.414).

Comparative statistical analysis between expression of bcl-2 and p53 in localized versus diffuse PVNS did not reveal any significant differences (p= 0.73).
Bcl-2 and p53 stainings were negative in all specimens with normal synovial tissue.

All analyzed cases stained positive for Ki-67 (Fig. 1c). A moderate Ki-67- proliferation rate (<10%, score 1) was seen in the majority of cases, both localized (91.3%) and diffuse PVNS (89.5%). A Ki-67 proliferation rate of 10-50% (score2) was found in 11,5% of diffuse PVNS and in 8.7% of localized PVNS. The differences were not statistically significant (p= 0.65).

All specimens with a Ki-67 proliferation rate of more than 10% (n=11) showed an expression of both p53 and bcl-2.

5 out of 20 samples of recurrent lesions originated from the same patients as the primary tumors (4 diffuse PVNS; 1 localized PVNS). These 5 cases were examined separately. An elevated expression of p53 was found in 60% of recurrent tumors (3/5). None of the primary lesions showed an elevated p53 expression

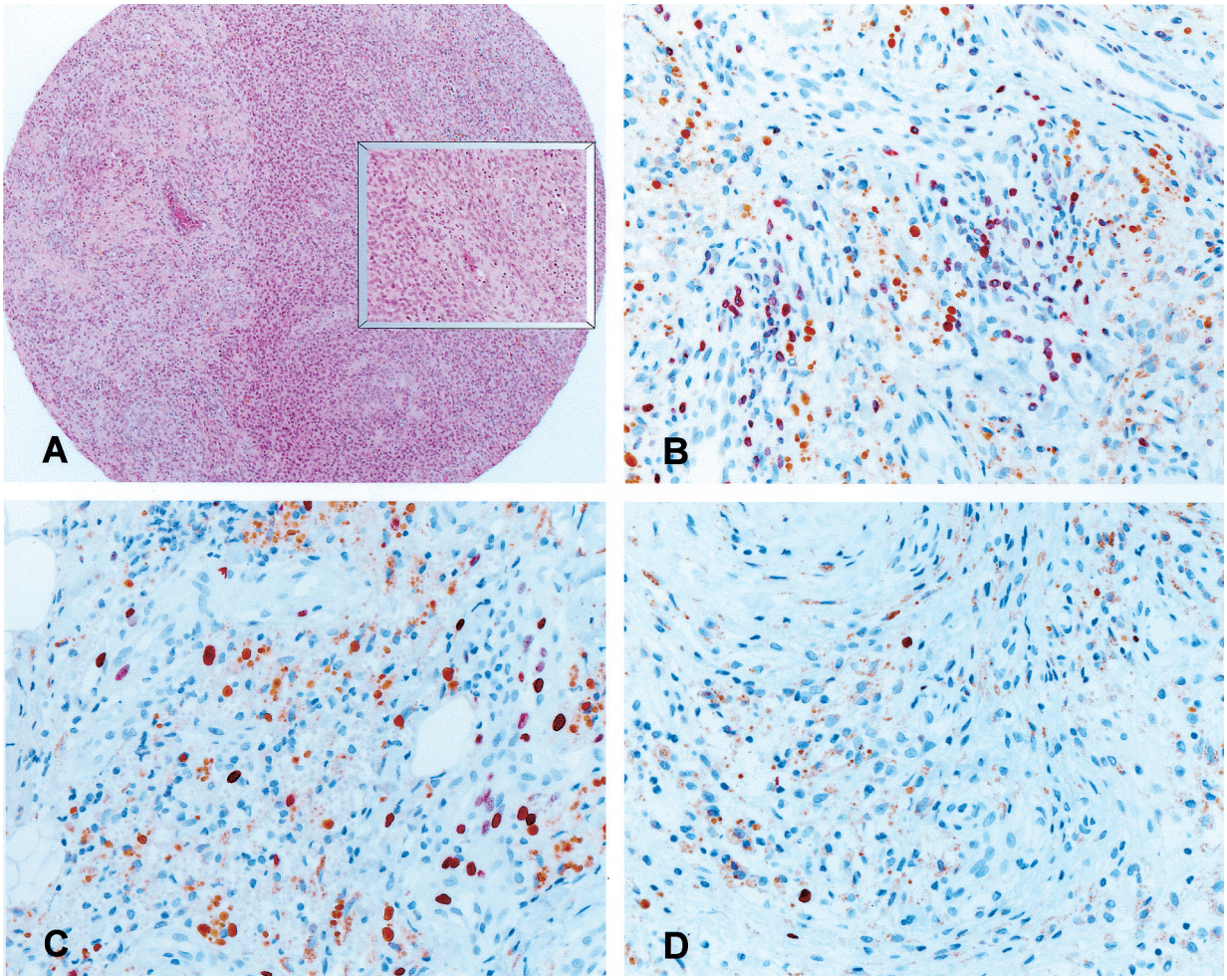

Fig. 1. Representative areas of synovial tissue with localised and diffuse PVNS are shown for: **A:** HE-stain of localized PVNS (tissue cylinder in tissue microarray). x 7.8. Insert x 26.0. **B.** Bcl-2 expression in diffuse PVNS. Positive cytoplasmatic staining (red) in numerous mononucleated synovial cells. Foamy cells and pigment-storing macrophages stain negatively. x 31.2. **C.** Ki-67 expression in diffuse PVNS. Positive nuclear staining (brown) in mononucleated synovial cells. Pigment-storing macrophages stain negatively. x 31.2. **D.** P53 expression in PVNS. Positive nuclear staining (brown) in singular mononucleated synovial cells. Pigment-storing macrophages stained negatively. x 31.2.

(p=0.17).

Bcl-2 expression and proliferation rate were similar in primary and recurrent PVNS (p=1.00).

Flow cytometry

43 cases (26 diffuse PVNS, 17 localized PVNS, including 5 primary and 5 recurrent PVNS lesions originating from the same patients) were investigated by flow cytometry for cell-cycle phase and DNA content. All cases with localized PVNS were diploid. All investigated cases with primary and recurrent PVNS were diploid. 3 out of 26 cases with diffuse PVNS were aneuploid (11.5%).

Most cells in analyzed PVNS cases were found to be in G0/1 phase fraction (84.3±7.18%) (Table 2). S-phase fraction in localised PVNS was 8.4±5.4% of cells (proliferation index 13.47±8.1). Diploid samples of diffuse PVNS showed S-phase fraction in 4.5±4.2% of cells (proliferation index 7.9±6.3). Aneuploid samples of the diffuse PVNS showed a significantly higher proliferative activity (S-phase fraction 11.8±8.6% (p=0.0545), proliferation index 16.4 ±14.1 (p=0.0580)).

5.51±4.46% of cells were found within the G2 phase of the cell cycle (proliferation index 9.3125±5.45). No significant differences in cell distribution within cell-cycle phases in primary versus recurrent lesions were found.

Discrimination of individual cells undergoing apoptotic death is possible by flow cytometry where apoptotic cells appear in a hypodiploid sub G0/1-peak as a consequence of partial DNA loss (Ehemann et al., 2003). No apoptotic cell fractions were detected in any investigated cases.

DNA fragmentation, expression of p53, caspase3 and bcl-2 and proliferative index were analyzed in 10 cases (5 primary and 5 recurrent PVNS originating from the same patients). DNA strand break was found in all investigated cases. DNA fragmentation was detected in 4.34±1.02% of cells in primary lesions and in 4.68±2.07% of cells in recurrent PVNS. The differences were not statistically significant (p=0.286).

Bcl-2 expression was found in 4.12±1.03% of cells in recurrent PVNS and in 3.18±1.57% of cells in primary lesions (p=0.905).

P53 expression was detected in 1.43±0.82% of cells in recurrent PVNS and in 1.56±0.87%of cells in primary lesions (p=1.0). Caspase3 expression was found in 2.05±0.61% of cells in recurrent PVNS and in 1.09±0.56% of cells in primary lesions (p=0.905) (Fig. 2).

A strong correlation between expression of bcl-2 and strand braces was detected in both primary and recurrent lesions (p=0.037). Expression of p53 correlated strongly

Table 2. Analysis of cell-cycle phases in primary and recurrent PVNS by flow cytometry.

CELL CYCLE PHASES	TOTAL (% of cells)	PRIMARY PVNS (% of cells)	RECURRENT PVNS (% of cells)
G0/1	90.3±5.43	89.7±7.78	90.5±2.31
S	4.98±3,31	6.08±3.03	4.19±3.65
G2	5.18±2.1	3.5±2.4	7.5±6.02
Proliferation index	9.31±5.45	8.50± 2.31	10.13±7.89

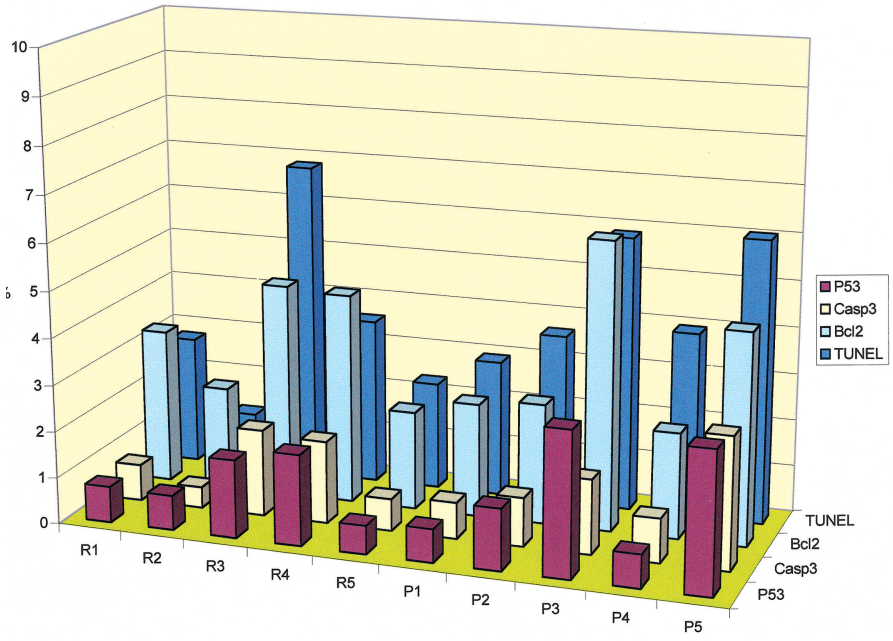

Fig. 2. Diagram representing data of multi-parametric flow cytometry (expression of p53, caspase3 and bcl-2, DNA fragmentation by TUNEL) in primary and recurrent PVNS originating from the same patients (% of positive-stained cells). P: primary PVNS; R: recurrent lesions 1-5 - case number.

with expression of caspase3 (p=0.0061) and with expression of bcl-2 (p=0.0013) but did not correlate with DNA fragmentation registered by TUNEL (p=0,108).

Discussion

In the present study we examined the role of the resistance to apoptosis as a possible mechanism leading to the sustained growth of PVNS. We examined the expression of bcl-2, p53 and Ki-67, the distribution of cell-cycle phases and analyzed DNA fragmentation in the different cell populations of these lesions.

Expression of the examined proteins was limited to the proliferating mononuclear synovial cells and was not detected in foamed cells, pigment-storing macrophages or giant cells. This finding is supported by a previous investigation demonstrating a lack of bcl-2 expression in osteoclast-like giant cells of bone and soft tissue tumors (Parmer et al., 1998). The authors postulate that these cells represent reactive infiltrates and are not of neoplastic origin. In PVNS, the proliferating mononucleated synovial cells appear be the only cell population with a resistance to the programmed cell death.

In contrast to normal synovial membrane cells, we observed an expression of bcl-2 in both localized and diffuse PVNS. Activation of bcl-2 has previously been associated with a cellular resistance to most apoptotic stimuli, suggesting that this protein functions at a central convergence of many apoptotic pathways (Charles et al., 1997). However, expression of bcl-2 was shown to immortalize cells without affecting cellular proliferation (Hockenberry et al., 1990; Wang and Reed, 1998). These data support our findings regarding the expression of bcl-2 and Ki-67. Concordant with previously published data (Adem et al., 2002), we observed a very low overall proliferative activity of PVNS independently of bcl-2 expression.

Up to 7% of cells involved in PVNS showed DNA fragmentations. In one part of these cells we found an elevated expression of p53, bcl-2 and caspase3 simultaneously. These cells may be undertaking DNA repair by activation of the p53-caspase pathway, a finding that is supported by a previous investigation suggesting that p53-mediated G1 arrest facilitates a prolonged interval for DNA repair (Jones and Wynford-Thomas, 1995). Flow-cytometric analysis gives further evidence towards this hypothesis by finding the vast majority of synovial cells in G0/1 phase (in some cases up to 93% of cells). However, bcl-2 expression and DNA fragmentations were detected in a significantly higher number of cells compared to the expression of p53 and caspase3. Since we did not observe any apoptotic cell fractions, cells with irrepairable DNA damage apparently escape the programmed cell death and accumulate in G0/1 phase.

Statistical analysis of data could not detect any significant correlations between any of the investigated parameters involved in apoptosis regulation or between age and sex of patients, affected joints, number of affected joints, duration of the symptoms, recurrence of disease and size of tumors. While apoptosis resistance seems to be a basic mechanism in pathogenesis of PVNS, expression of p53, bcl-2, caspase3, proliferative activity (Ki-67) as well as level of DNA fragmentation are not useful as prognostic or predictive factors in progression or recurrence of PVNS.

In conclusion, apoptosis resistance in PVNS is limited to the proliferating synovial cells and may essentially contribute to the survival of these cells, leading to a permanent slow progression of the lesions.

Irrespective of the question as to whether PVNS is of neoplastic or inflammatory origin, we postulate that apoptosis resistance is a critical event in this lesion. A better understanding of the mechanisms responsible for synovial cell apoptosis may contribute to the development of new therapeutic strategies in PVNS.

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