Synovial colony-stimulating factor-1 mRNA expression in diffuse pigmented villonodular synovitis

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SUMMARY

Objective. *To delineate the molecular mechanisms underlying the process of the diffuse-type giant cell tumours, also called pigmented villonodular synovitis, a rare, aggressive condition of the synovium, the knee synovial tissue expression of colony-stimulating factor-1 gene, as detected by real-time polymerase chain reaction, was compared between patients affected with pigmented villonodular knee synovitis and knee meniscal tears, or persistent gonoarthitis.*

Methods. *Multiple synovial biopsies of the knee were performed by arthroscopy in five consecutive patients affected by diffuse pigmented villonodular knee synovitis and in 12 patients affected by knee meniscal tears (n. 6) or persistent active gonarthritis (n. 6), recruited from the patients attending the Rheumatology Day Surgery Outpatient Clinic of the University of Padova Hospital. The ethics committee approved the study protocol and the participants signed consent statements after being informed about the content of the study. The diagnosis was made on the basis of a histological examination.*

The colony-stimulating factor-1 gene expression was assessed by reverse transcription followed by real-time polymerase chain reaction.

Results. *The detection by RT-PCR of synovial colony-stimulating factor-1 mRNA showed a wide spectrum of expression in the three groups of distinct knee joint disease affected patients, with significantly higher level of colony-stimulating factor-1 mRNA expression in synovial tissue of pigmented villonodular synovitis, in comparison to that of knee meniscal injuries and persistent gonoarthritis patients.*

Conclusions. *Our findings point out to an important role of colony-stimulating factor-1 in pigmented villonodular knee synovitis disease process and support the idea that colony-stimulating factor-1/colony-stimulating factor-1 receptor interaction may represent a potential therapeutic target of this disease.*

Introduction

Tenosynovial giant cell tumours (TSGCT) are benign mesenchymal tumours of the synovial lining. Diffuse-type giant cell tumours, also called pigmented villonodular synovitis (PVNS), is a rare, predominantly intra-articular and aggressive infiltrative condition of the synovium primarily affecting large joints (1, 2). Histologically, PVNS consists of synovial-like histiocytes and multinucleated giant cells (MGCs), lymphocyte infiltrates, siderophages and lipid-loaded macrophages (1, 2). It has been reported that PVNS patients are at considerable risk of multiple local recurrence and of local destructive course.

The inflammatory nature of PVNS has been suggested by the immunohistochemical (IHC) marker expression by the synovial-like membrane consisting of areas containing monocyte/macrophages: CD68, CD163, CD55, matrix metalloproteinase (MMP) MMP-2,-9, colony-stimulating factor-1 (CSF-1), CSF-1 receptor (CSF-1R), receptor activator for nuclear factor κB ligand (RANKL), typical of activated macrophages as well as the IHC phenotype of PVNS-MGCs: RANKL, CSF-1R, CD33, CD51, tartrate-resistant acid phosphatase (TRAP), calcitonin-receptor, characteristic of osteoclasts (3-8).

A neoplastic origin of TSGCTs has, instead, been supported by the finding of up-regulated telomerase activity, by the identification of DNA aneuploidy and of clonal karyotypic aberrations (4, 9, 10), and, more recently, by the observation of frequent chromosomal translocations involving CSF-1 locus and collagen type VI alpha-3 (COL6A3) genes in TSGCTs/D-TSGCT cells 12 (11).

CSF-1 acts through CSF-1R, a tyrosine kinase receptor, encoded by the c-*fms* protooncogene (12), primarily expressed on mononuclear phagocytic cells (13). Up-regulation of CSF-1 has already been reported in chronic inflammatory diseases and in cancer (14-16).

The study was undertaken as part of a multidisciplinary collaborative study to explore new intraarticular therapeutic approach and synovial molecular targets in PVNS. To this end, the molecular mechanisms of synergic paracrine loop mediated by TNF-α and CSF1 were explored in both inflammatory and neoplastic conditions (17).

The present study was designed with the intent of delineating the mole-

cular mechanisms underlying the PVNS process by assessing CSF-1 messenger RNA (mRNA) expression in knee synovial tissue by real-time polymerase chain reaction (RT-PCR) and by comparing these values in patients affected with diffuse PVNS, with knee meniscal tears or persistent gonoarthitis.

Patients and methods

Five consecutive patients affected with diffuse knee PVNS (the diagnosis was made on the basis of a histological examination) (5 knees) (4 male and 1 female) (age-range 20–35 years), characterised by persistent warmth, tenderness, swelling and effusion, proving to be resistant to repeat intraarticular corticosteroid injections, and six patients (6 knees) (4 male and 2 female) (age-range 38–51 years) affected with persistent active gonarthritis despite undergoing >2 intraarticular corticosteroid injections during the year before (RM imaging showed diffuse knee joint synovial proliferation) were recruited from the patients attending the Rheumatology Outpatient Clinic of the University of Padova Hospital. All the patients were over 18 years of age. Six more consecutive patients over the age of 18 and affected with knee meniscal injuries (6 knees) (3 male and 1 female) (age-range 34–41 years), who were submitted to knee joint arthroscopic meniscectomy at the Multidisciplinary Outpatient Clinic of the University of Padova Hospital, were also studied (Table I).

The ethics committee approved the study protocol (Etanercept/TNR-001: n. 878P) and the participants signed consent statements after being informed about the content and methodology of the study.

Synovial biopsy

Synovial biopsies of the knee were performed during arthroscopy carried out in our Department. The synovial specimens were obtained targeting the areas of intense synovial hyperemic proliferation. Multiple biopsy samples from each patient were preserved both in RNA-later and in paraformaldehyde and embedded en bloc in paraffin. Paraffin bloc sections (5 mm) were cut for histologic analysis. The synovial membrane tissue specimens were stained with haematoxylin and eosin (H&E).

Total RNA extraction

Total RNA was extracted from frozen biopsies using a commercially available kit (RNA Ble, RNA Extraction, Eurobio, France). The extracted RNA had a 280/260 OD ratio between 1.8 and 2.0 and RNA integrity was evaluated by agarose gel electrophoresis.

Real-time PCR analysis of CSF-1 mRNA expression

Gene expression of the components of the CSF-1 was assessed by reverse transcription followed by real-time polymerase chain reaction (PCR; TaqMan; PE Applied Biosystems, Foster City, CA, USA). Briefly, RNA reverse transcription was performed using the Gene Amp RNA PCR Kit, essentially following the manufacturer's directions (Applied Biosystems, Foster City, CA,

USA). Briefly, RNA (~1 μg) was reverse transcribed using random hexamer primers and MuLV reverse transcriptase in a GeneAmp PCR System 2700 (Applied Biosystems) and in accordance with the following protocol: 15 min at 42°C, 5 min at 99°C and 5 min at 5°C.

Real-time polymerase chain reaction (RT-PCR) was carried out in an ABI Prism 7000 sequence detection system using *Taq*Man Universal PCR Master Mix, primers and probes mapping a common sequence of CSF-1 isoforms and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) purchased from Applied Biosystems. After treatment with Uracil-N-Glicosylase (UNG) for 2 min at 50°C to minimise DNA contamination, and inactivation of this enzyme at 95°C for 10 min, the samples were amplified with the following protocol for 45 cycles: denaturation 95°C for 15 s, and annealing/extension 60°C for 1 min. Each PCR amplifica-

Table I. Clinical and demographic characteristics of patients affected with (**A**) diffuse pigmented villonodular knee synovitis, (**B**) persistent active gonoarthritis and (**C**) knee meniscal tears.

A) Patients affected with diffuse pigmented villonodular knee synovitis

n.	Age	Sex	Side	Synovitis duration (years)	Disease duration (years)
1	35	F	L	4	12
2	30	M	L	4.5	4.5
3	32	M	R	4	8
4	31	M	L	5	5.8
5	20	M	L	2	2
mean	29.6			3.90	6.46
SD(±)	5.68			1.14	3.78

B) Patients affected with persistent active gonoarthritis

n.	Age	Sex	Side	Gonoarthritis duration (years)	Disease duration (years)
1	44	M	L	11.5	13.5
2	40	F	L	8.9	9.1
3	40	F	L	8.9	9.1
4	44	M	L	11.5	13.5
5	51	M	L	5.7	22
6	38	M	R	6.4	8.4
mean	42.83			8.82	12.60
SD(±)	4.67			2.45	5.14

C) Patients affected with knee meniscal tears

n.	Age	Sex	Side	Knee meniscal tears duration (years)	Disease duration (years)
1	40	F	L	2	4
2	41	M	R	1.8	3
3	34	F	L	2	2.9
4	36	M	L	1.5	3
5	35	M	R	1	1.4
6	38	F	R	3	4
mean	37.33			1.88	3.05
SD(±)	2.80			0.66	0.95

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Table II. CSF-1 mRNA expression by real-time PCR in synovial knee joint specimens obtained in diffuse pigmented villonodular knee synovitis, persistent active gonoarthritis and knee meniscal tears affected patients. CSF-1 mRNA expression was estimated from the ratio of fluorescence intensity to GAPDH.

n.	CSF-1/GAPDHmRNA		
	Knee meniscal tears	Gonoarthritis	PVNS
1	0.97	14.05	22.91
2	8.71	18.98	25.14
3	0.84	17.87	24.61
4	0.76	13.13	22.98
5	1.85	18.3	25.44
6	7.94	16.39	
mean	3.51	16.45	24.22
SD(±)	3.76	2.39	1.20

tion was performed in duplicate. A pool of standard cDNA was used in a serial dilution of 1, 1:5, 1:25 and 1:125 for standard curves. A non-template (water) reaction mixture was prepared as a negative control. CSF-1 mRNA expression was estimated from the ratio of fluorescence intensity to GAPDH.

Statistical analysis
Statistical analysis was performed with SPSS software. Results were analysed using the non-parametric Kruskal-Wallis test. Pairwise group comparisons were made by Mann-Whitney test for unpaired samples followed by Bonferroni correction (18). *P*-values <0.01 were considered significant.

Results
Histology
All the PVNS H&E stained synovial membrane tissue specimens showed moderate proliferation of the synovial lining, mononuclear cell infiltration, diffuse siderosis, intense stromal vascularity and extracellular matrix deposition.

Synovial CSF-1 mRNA expression
The detection by RT-PCR of synovial colony-stimulating factor-1 mRNA showed a wide spectrum of expression in the three groups of distinct knee joint disease affected patients, with significantly higher level of colony-stimulating factor-1 mRNA expression in synovial tissue of pigmented villonodular synovitis, in comparison to that of knee meniscal injuries and persistent gonoarthritis patients (Table II; Fig. 1).

Discussion
The results of this preliminary study are consistent with previous findings of high synovial CSF-1 expression by *in situ* hybridisation in PVNS patients (19, 20) and indicate that CSF-1 may also play a role in persistent inflammatory gonarthritis (19, 20). Moreover, to

our knowledge, the direct comparison of CSF-1 expression between PVNS and meniscal injurie or gonarthritis, was previously unreported.

It was thought that the translocation of CSF-1 in PVNS limited to the neoplastic cells led to the recruitment of the majority of reactive non-neoplastic CD68^{+} macrophages (11, 21, 22). The recent finding of high CSF-1 expression in translocation-negative PVNS patients implies that alternative mechanisms may lead to up-regulation of CSF-1 in these patients (23).

Synergistic activity of TNF-α and CSF-1 was found to be involved in monocyte activation, macrophage proliferation (24, 25) and osteoclast differentiation (26, 27). A paracrine synergistic loop, mediated by TNF-α and CSF-1 was indeed demonstrated in both inflammatory (20, 27, 28) and neoplastic conditions (16, 29), playing an important role in the regulation of differentiation/proliferation of mononuclear phagocytes and osteoclasts (26, 27), modulation of dendritic cells' (DCs) development and acceleration of angiogenesis and neovascularisation (29-32). Inflammatory or cancer cell–derived TNF-α has been shown, in fact, to stimulate TNF-α and CSF-1 production by macrophages, and CSF-1, in turn, induces macrophage VEGF-A and MMP-2 in an autocrine manner (16).

The exiguous number of patients studied by us is an important limitation of this study. Larger, controlled studies are, therefore, clearly warranted to further assess the clinical relevance of synovial CSF-1 in diffuse PVNS and the potential utility of CSF-1 mRNA expression as diagnostic biomarkers in the early phases of the disease.

Understanding the molecular pathways involved in the PVNS process will undoubtedly open the door to the development of new therapies targeting molecular alterations (33-35). Recent studies concerned with the biological effects of CSF-1 and of CSF-1R block (36-38) have produced promising findings both in inflammatory (39, 40) and neoplastic conditions (41-43).

Previous reports (11, 21, 22, 17), our preliminary findings concerning CSF-1 up-regulation in PVNS, and prelimi-
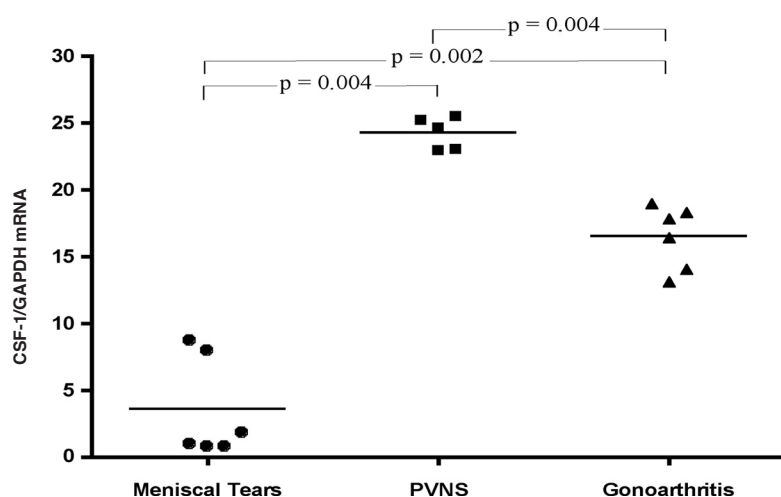
Fig. 1. CSF1-mRNA levels detected by real-time PCR in frozen samples of knee synovial tissues biopsies obtained from knee meniscal tears (n=6), gonarthritis affected patients (n=6) and diffuse pigmented villonodular knee synovitis (n=5).
Results were analysed using the non-parametric Kruskal-Wallis test (*p*=0.0008) followd by Mann-Whitney test with Bonferroni correction. All comparisons were statistically significant.
Horizontal lines represent mean value for each group.
CSF-1 mRNA expression was estimated from the ratio of fluorescence intensity to GAPDH.

nary data on imatinib for the systemic therapy of a patient with PVNS (44), seem to indicate that CSF1/CSF1R interaction may represent a potential therapeutic target.

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