Aberrant Collagenase Expression in Chronic Idiopathic Myelofibrosis Is Related to the Stage of Disease but Not to the JAK2 Mutation Status

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Bone marrow fibrosis in chronic idiopathic myelofibrosis (cIMF) most likely represents an imbalance between synthesis and turnover of collagen fibers. Because the JAK-STAT signaling pathway is involved in the regulation of genes encoding matrix metalloproteinases (MMPs), we examined the expression of MMPs, their tissue inhibitors (TIMPs), and collagen types in relation to the JAK2 status (V617F mutation versus wild-type) in cIMF ($n = 64$). Whereas no correlation was found between the JAK2 status and MMP gene products, there was an evident association with the stage of disease. Membrane type 1-MMP (MMP-14) was overexpressed by up to 80-fold in advanced stages that progressed to fibrosis ($P < 0.001$), and megakaryocytes and endothelial cells were unmasked as the major cellular source. By contrast, a significantly higher expression of neutrophil collagenase (MMP-8) was encountered in the prefibrotic stages of cIMF ($P < 0.001$). Altogether, the stepwise progress of myelofibrosis in cIMF was associated with expression of a defined subset of target genes as shown in sequential trephine biopsies of cIMF patients. We conclude that the expression of matrix-modeling genes in cIMF is not influenced by the JAK2 mutation status but is predominantly related to the stage of disease. (*Am J Pathol* 2006; 169:471–481; DOI: 10.2353/ajpath.2006.060110)

According to the World Health Organization classification, Philadelphia chromosome-negative chronic myeloproliferative disorders comprise polycythemia vera, essential thrombocythemia, and chronic idiopathic myelofibrosis (cIMF) along with more rare entities such as chronic neutrophilic leukemia and chronic eosinophilic leukemia/hypereosinophilic syndrome.¹ cIMF shows pro-

gressive bone marrow fibrosis, increased angiogenesis, enhanced trafficking of CD34⁺ cells with extramedullary hematopoiesis and greater risk for transformation into acute leukemia.² In up to 50% of patients, mutated and therefore constitutively activated JAK2 has been demonstrated to be the underlying molecular defect responsible for autonomous proliferation.^{3–7}

The extracellular matrix (ECM) is a complex meshwork comprising various types of collagens, laminin, entactin, heparan sulfate, and proteoglycans.⁸ The ECM is substantially involved in the interaction of hematopoietic cells and bone marrow stroma cells.⁹ In normal bone marrow, resident fibroblasts are responsible for constitutively synthesized collagens.¹⁰ Patients with cIMF either present with manifest myelofibrosis or present with a prefibrotic, cellular phase with no or only minute degrees of collagen fiber increase in the bone marrow.^{11,12} The onset of progressive deposition of collagens in prefibrotic cIMF is variable. Collagen type-3 (COL-3, also termed reticulin) and collagen type-1 (COL-1), synthesized by nonclonal fibroblasts, seem to be the predominant ECM components in the process of myelofibrosis.²

The family of matrix metalloproteinases (MMPs) is comprised of more than 20 enzymes that can collectively degrade all components of the ECM.¹³ MMP-1, -8, -13, and -14 (MT1-MMP) are thought to be the most potent collagenolytic MMPs.¹³ Of note, MMP-1, -8, and -13 cleave the triple helix of collagens, thereby allowing the chains to unwind and become susceptible to further degradation by other MMPs. MMP-2 is a gelatinase but, in contrast to MMP-9, is also highly potent in the degradation of collagen types I, II, and III.¹³ It appears likely that an imbalance of ECM synthesis and activation of proteolytic enzymes paves the way for the increasing collagen meshwork in cIMF. Conversely, stem cell trafficking and

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Table 1. Brief Clinical Description of the Study Group

cIMF stage	No.	Age	Sex F/M	Leukocytes (10 ³ /μl)	Platelets (10 ³ /μl)	Erythrocytes (10 ⁶ /μl)	Hemoglobin (g/dl)
Cellular cIMF	31	68 (42 to 89)	17/14	12.7 (7.2 to 116.4)	767 (250 to 1607)	4.5 (3.1 to 6.3)	13.4 (7.4 to 16.6)
Advanced cIMF	33	72 (37 to 89)	15/18	10.1 (1.9 to 42.5)	351 (63 to 1214)	3.7 (2.7 to 6.6)	10.8 (5.3 to 15.6)

Median values are followed by the range. F, female; M, male.

angiogenesis as hallmarks in advanced cIMF stages require local matrix turnover, ie, proteolytic action, for full establishment.¹⁴ It has been demonstrated that the JAK-STAT pathway is involved in the activation of genes responsible for synthesis and secretion of MMPs.^{15,16} Besides revealing the role of JAKs and MMP activity in the physiological processes of hematopoiesis and differentiation, recruitment of this particular pathway has also been demonstrated during tumor invasion and neoangiogenesis.^{17–20} We tested the hypothesis of a potential correlation of an underlying JAK2 mutation in cIMF that leads to aberrant expression of MMPs, TIMPs, and COLs in different disease stages and during the course of the disease.

Materials and Methods

Bone Marrow Study Group

Formalin-fixed and paraffin-embedded (FFPE) bone marrow trephines with proven cIMF were retrieved from the bone marrow registry of the Institute of Pathology, Hannover Medical School. Bone marrow trephines were routinely fixed in a solution containing phosphate-buffered formalin (pH 7.4) for 24 hours. The decalcification step was performed in an ethylenediaminetetraacetic acid-based solution (pH 7.5) for up to 48 hours. The study group ($n = 88$) comprised cellular, prefibrotic cIMF ($n = 31$), advanced cIMF with manifest myelofibrosis ($n = 33$), and 24 control cases showing normal hematopoiesis. Based on the World Health Organization classification and in close agreement with clinical data and presentation, patient bone marrow trephines were initially diagnosed to have a cIMF in the years 2000 to 2004. In addition, sequential bone marrow trephines in two patients were investigated, representing follow-ups of 3 years. cIMF cases were re-evaluated and subdivided into two groups depending on the degree of myelofibrosis after silver impregnation (Gomori) as described.^{11,21} For a summary of patient clinical data, see Table 1.

Real-Time Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR)

As we previously described,²² total RNA was extracted from total FFPE bone marrow cells after guanidinium isothiocyanate/proteinase K-based digestion and conventional organic extraction using phenol/chloroform. Total RNA (1 μg), pretreated with RNase-free (RNase[−]) DNase (1 U/μg RNA, RQ1; Promega, Madison, WI), was transcribed into the complementary DNA using 500 ng of random hexamers (Amersham Pharmacia, Piscataway,

NJ) and 200 U of SuperScript II RNase[−] reverse transcriptase (Invitrogen, Karlsruhe, Germany) in a volume of 20 μl following the manufacture's protocol. Negative controls were performed by using water instead of reverse transcriptase. Real-time PCR was performed on an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). For sequences of PCR primers and TaqMan probes that amplify COL-1, COL-3, MMP-2, MMP-8, MMP-13, MT1-MMP, TIMP-1, TIMP-2, and the housekeeping gene β -glucuronidase see Table 2. The real-time PCR amplification was performed in a final reaction volume of 25 μl containing primers (250 nmol/L each), probe (150 nmol/L), 0.5 U of Platinum Taq polymerase (Invitrogen), 200 μmol/L each of dATP, dCTP, dTTP, and dGTP in 1× Platinum Taq reaction buffer and 4 μl of cDNA. The reaction mixture was preheated at 95°C for 5 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. Amplification for COL-1, COL-3, MMP-2, MMP-8, MMP-13, MMP-14, TIMP-1, TIMP-2, and β -glucuronidase could be demonstrated to be linear throughout a broad concentration range, allowing relative quantification in two independent runs using the $\Delta\Delta C_T$ method as described.^{23,24} Briefly, the relative transcript level was expressed as the difference of the C_T values: C_T [target gene, control group] – C_T [housekeeping gene, control group] = ΔC_T [control group]. To determine the case-specific gene expression in the control group, gene expression was correlated for every single control case relative to the mean value [$=\Delta C_T$ (control group)]. The target gene expression in different disease stages of cIMF was calculated accordingly as described above: C_T [target gene, cIMF case no. 1, 2, 3, . . .] – C_T [housekeeping gene, cIMF case no. 1, 2, 3, . . .] = ΔC_T [cIMF case no. 1, 2, 3, . . .]. To determine the case-specific gene expression in cIMF, every single case of a disease stage, ie, prefibrotic cIMF versus advanced cIMF, was calculated relative to the mean ΔC_T [control group] as follows: fold change = $1.9^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ stands for: ΔC_T [cIMF case no. 1, 2, 3, . . .] – ΔC_T [control group]. For example: $\Delta\Delta C_T = -3.5$ means that this case expressed 9.5-fold more transcripts ($1.9^{-(-3.5)}$) compared to the control group. For a comprehensive review of the equations underlying this quantification algorithm, see Livak and Schmittgen.²³

DNA Extraction, JAK2 Amplification, and Restriction Site Analysis

The hotspot guanine-to-thymine point mutation in exon 12 of JAK2 destroys a relevant recognition site for the restriction enzyme *BsaXI* and avoids digestion of amplified

Table 2. Primer and Probes Used Throughout This Study

Primer and probes	Sequence	Reference/product
COL1 forward	5'-AAACTTTGCTCCCCAGCTGTC-3'	
COL1 reverse	5'-CGAGGACCAGAGGGACCC-3'	
COL1 probe	5'-FAM-TATGGCTATGATGAGAAATCAACCGGAGGAATTT-TAMRA-3'	GenBank NM 000088, 90 bp
COL3 forward	5'-CAGGATACCAAGGACCCCT-3'	
COL3 reverse	5'-TGGACCTATAGCACCAGGAGGT-3'	
COL3 probe	5'-FAM-TGAACCTGGGCAAGCTGGTCCTTC-TAMRA-3'	GenBank NM 000090, 80 bp
MMP-1 forward*	5'-GCAAACACATCTGACCTACAGGATT-3'	
MMP-1 reverse*	5'-CTTTCTCAATGGCATGGTCCA-3'	GenBank NM 002421, 80 bp
MMP-1 forward†	5'-ACGGATACCCCAAGGACATCT-3'	
MMP-1 reverse†	5'-TCAGAAAGAGCAGCATCGATATG-3'	GenBank NM 002421, 76 bp
MMP-2 forward	5'-ATGCCGTCGTGGACCTGC-3'	
MMP-2 reverse	5'-TTCACGCTCTTCAGACTTTGGTTC-3'	
MMP-2 probe	5'-FAM-CAGCTTCAGGTAATAGGCACCCTGAAGAAGTAGC-TAMRA-3'	GenBank AY 738117, 94 bp
MMP-8 forward	5'-AGCGATTTTTTGGGTGAATGTG-3'	
MMP-8 reverse	5'-AAACCACCACTGTCAGGCACTC-3'	
MMP-8 probe	5'-FAM-AGCCAAATGAGGAACTCTGGACATGATGAAA-TAMRA-3'	GenBank NM 002424, 97 bp
MMP-13 forward	5'-AGGAAGACCTCCAGTTTGCAGAG-3'	
MMP-13 reverse	5'-GCTGCATTCTCCTTCAGGATT-3'	
MMP-13 probe	5'-FAM-TACCTGAGATCATACTACCATCCTACAAATCTCGCG-TAMRA-3'	GenBank NM 002427, 85 bp
MMP-14 forward	5'-TGGAGGAGACACCCACTTTGA-3'	
MMP-14 reverse	5'-GCCACCAGGAAGATGTCATTTTC-3'	
MMP-14 probe	5'-FAM-TCTGCCGAGCCTTGGACTGTCAGG-TAMRA-3'	GenBank NM 004995, 84 bp
TIMP-1 forward	5'-ACTGTTGGCTGTGAGGAATGC-3'	
TIMP-1 reverse	5'-CACAAGCAATGAGTGCCACTCT-3'	
TIMP-1 probe	5'-FAM-TTCCCTGTTTATCCCTGCCACTGCAAACT-TAMRA-3'	GenBank AY 932824, 80 bp
TIMP-2 forward	5'-AGAAGAAGAGCCTGAACACAG-3'	
TIMP-2 reverse	5'-ATGTAGCACGGGATCATGGG-3'	
TIMP-2 probe	5'-FAM-ACCAGATGGGCTGCGAGTGCAAGAT-TAMRA-3'	GenBank NM 003255, 79 bp
β-GUS forward	5'-CTCATTGGAAATTTGCCGATT-3'	
β-GUS reverse	5'-CGAGTGAAGATCCCCTTTTA-3'	
β-GUS probe	5'-FAM-TGAACAGTCACCGACGAGAGTGCTGG-TAMRA-3'	GenBank NM 000181, 81 bp

*Custom made.

†Higashikata T, Yamagishi M, Sasaki H, Minatoya K, Ogino H, Ishibashi-Ueda H, Hao H, Nagaya N, Tomoike H, Sakamoto A: Application of real-time RT-PCR to quantifying gene expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in human abdominal aortic aneurysm. *Atherosclerosis* 2004, 177:353–360.

JAK2 as described.⁵ We adopted this methodology for analysis of FFPE bone marrow cells.²⁵ For DNA extraction from total bone marrow cells, one ~10-μm slide was cut from the FFPE block before application of the DNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. PCR amplification of JAK2 was performed for 40 cycles in a GeneAmp PCR System 2700 (Applied Biosystems, Weiterstadt, Germany) using 2.5 mmol/L magnesium chloride and 25 ng of DNA. Primers for JAK2 amplification covering the hotspot mutation site corresponding to nucleotide 55.061 (GenBank sequence AL161450) were: JAK2 forward 5'-TATGATGAGCAAGCTTTCTACAAG-3', and JAK2 reverse 5'-AGAAAGGCATTAGAAAGCCTGTAGTT-3' generating a 102-bp product.

Ten μl of a given sample were incubated with 4 U of the restriction enzyme *Bsa*XI (2 U/μl; New England Biolabs, Beverly, MA) for 6 hours at 37°C in a PCR cyclor with heated lid to avoid sample evaporation. The *Bsa*XI enzyme recognized a site (5' . . . ₉(N) A C (N)₅ C T C C (N)₁₀ . . . 3'), which included the hotspot nucleotide 55.061 (GenBank sequence AL161450). For determination of *Bsa*XI digestion in the respective JAK2 sequence, the NEBcutter 2.0 software tool was used (<http://tools.neb.com/NEBcutter2/index.php>). Samples analyzed by restriction site analysis were strictly accompanied by a

positive control (JAK2 mutated cell line HEL) and a negative control (normal hematopoiesis).

JAK2 (V617F) Genotyping and Allele Quantification by Pyrosequencing

We established a pyrosequencing assay to quantify the percentage of mutant alleles in bone marrow cells from different disease stages of cIMF. The 102-bp fragment covering the hotspot point mutation (1849G>T) was amplified by PCR from 25 ng of genomic DNA with the primer combination used for *Bsa*XI restriction site analysis with an additional 5'-biotin tag in a final volume of 50 μl. Forty-five μl of PCR product were mixed for 5 minutes (1200 rpm) at room temperature with 3.0 μl Streptavidin Sepharose HP (Amersham Biosciences, Freiburg, Germany) and 47 μl of binding buffer (Biotage, Uppsala, Sweden) using a thermomixer (Eppendorf, Hamburg, Germany). Using the Vacuum Prep tool (Biotage), single-stranded PCR products were prepared for sequencing analysis. Templates attached to the beads were washed with 70% ethanol for 5 seconds, denatured in 0.5 mol/L NaOH solution for 10 seconds, and washed in washing buffer (Biotage) for 5 seconds. Then the vacuum was switched off, and the beads were released into a PSQ 96

Plate Low (Biotage) containing 45 μ l of annealing buffer (Biotage) and 500 nmol/L sequencing primer (5'-GGTTT-TAAATTATGGAGTATGT-3', nucleotides 55.039 to 55.060 in GenBank AL161450).

The samples were heated to 80°C for 2 minutes and then cooled to room temperature. Pyrosequencing reactions were performed in a PSQ 96MA instrument (Biotage) according to the manufacturer's instructions using the PyroGold SNP reagent kit (Biotage), which contains enzyme, substrate mixture, and nucleotides. Allele frequency was quantified using the SNP software (Biotage). As described, samples were scored as heterozygous for the JAK2 mutation if the percentage of mutant alleles exceeded 5%.²⁶ Homozygosity was considered if the percentage of mutant T alleles exceeded 50%.⁴

Immunohistochemistry

To delineate cellular sources for MMP-14 (MT1-MMP), bone marrow showing cellular, prefibrotic cIMF ($n = 10$), advanced cIMF ($n = 11$), and control cases representing either normal hematopoiesis ($n = 11$) or reactive megakaryocytic hyperplasia ($n = 10$) were stained with a monoclonal anti-human MMP-14 antibody (MAB918; R&D Systems, Minneapolis, MN) recognizing the catalytic domain of the mature peptide. Bone marrow sections ($\sim 2 \mu$ m) were stained with the Ready-to-use (Vector Laboratories). Vectastain ABC kit and the peroxidase DAB substrate kit (no. PK-7200 and no. SK-4100, respectively; R.T.U., Burlingame, CA) according to the manufacturer's instructions. Briefly, sections were pretreated in citrate buffer, pH 6.0, in a microwave oven at 900 W and 750 W, 10 minutes each. Endogenous peroxidase was blocked by incubation with 3% H_2O_2 at room temperature for 10 minutes followed by incubation with normal horse serum for 1 hour at room temperature. Sections were incubated overnight with the primary anti-human MMP-14 antibody at 4°C. After overnight incubation the sections were incubated with the secondary antibody and ABC components for 30 minutes each. DAB staining was performed for 2 minutes at room temperature followed by counterstaining using hemalum according to standard protocols. FFPE colon carcinoma tissue (R&D Systems) served as the positive control according to the distributor's instructions. Negative controls were performed for each staining procedure by omitting the secondary antibody.

Statistical Analysis

To analyze differences of gene expression in cellular prefibrotic cIMF, advanced cIMF, and nonneoplastic hematopoiesis, nonparametric Kruskal-Wallis tests were performed followed by Mann-Whitney *U*-tests for pairwise group differences (*P* values Bonferroni-adjusted). Kruskal-Wallis tests were also performed to analyze differences of gene expression in V617F JAK2-mutated and wild-type JAK2 in both cellular cIMF and advanced cIMF. *P* values ≤ 0.05 were considered as statistically significant.

Table 3. JAK2 Mutation Status in the Study Group

cIMF stage	Homozygosity for V617F JAK2	Heterozygosity for V617F JAK2	JAK2 wild type
Cellular cIMF	9%	36%	55%
Advanced cIMF	13%	40%	47%

Results

Comparison of JAK2 Mutation Status and Expression of Genes Involved in the Turnover of Fibers

The overall frequency of V617F JAK2 mutations in the study group was 45% (9% homozygosity, 36% heterozygosity) in cellular cIMF and 53% (13% homozygosity, 40% heterozygosity) in advanced cIMF. Control cases under study showed entirely wild-type JAK2 (Table 3). Heterozygous V617F JAK2, homozygous V617F JAK2, and wild-type JAK2, as evidenced by our pyrosequencing assay, are shown in Figure 1. Restriction site analysis and pyrosequencing rendered identical results. The JAK2 mutation status or the wild-type state did not correlate with expression of MMPs, TIMPs, and COLs except for TIMP-2, which showed a higher level in cellular cIMF cases with wild-type JAK2 ($P = 0.003$). A comprehensive analysis of potential correlation of JAK2 status and gene expression is summarized in Table 4.

Aberrant Expression of Collagenase Subsets (MMP-8, MMP-13, MMP-14/MT1-MMP) Define Different Stages of Disease in cIMF

MMP-14 mRNA was demonstrated to be significantly overexpressed in advanced cIMF ($n = 33$) by up to sevenfold (median, 2.4; range, 0.5 to 7.1) compared to prefibrotic cIMF (median, 0.9; range, 0.3 to 2.0; $P < 0.001$; $n = 31$) and control hematopoiesis (median, 0.8; range, 0.5 to 2.0; $P < 0.001$; $n = 17$) (Figure 2F). MMP-13 mRNA levels showed differences between advanced cIMF (median, 4.6; range, 0.5 to 83.2; $n = 32$) and the cellular, prefibrotic phase (median, 0.7; range, 0.2 to 2.4; $P < 0.001$; $n = 23$). Normal hematopoiesis (median, 1.2; range, 0.2 to 4.3; $n = 16$) did not differ from either cIMF stage (Figure 2E). MMP-8 mRNA was significantly up-regulated in cellular, prefibrotic cIMF ($n = 25$) by up to sixfold (median, 2.2; range, 0.3 to 6.0) as compared to control hematopoiesis (median, 1.0; range, 0.3 to 2.8; $P < 0.001$; $n = 24$). Cellular cIMF and advanced cIMF (median, 1.2; range, 0.5 to 11.5; $n = 15$) did not differ significantly from each other (Figure 2D).

Significant Up-Regulation of Collagen Genes Defines the Advanced Stage in cIMF

COL-3 mRNA was significantly increased in advanced cIMF by up to 76-fold (median, 5.3; range, 1.0 to 76.3;

Figure 1. Genotyping analyses by the V617F JAK2 pyrosequencing assay are shown. The heights of peaks as shown in the pyrogram traces are proportional to the amount of nucleotide in the sequenced DNA. The mutation site is displayed in yellow and represents the potential hot spot and an adjacent normal T. Pyrosequencing of a case heterozygous for the V617F JAK2 mutation showed 60% G and 40% for the mutant T allele (**A**); a case homozygous for the mutation showed ~13% G and 87% for the mutant T allele (**B**). A wild-type control case showed 100% G and no mutant T allele at all (**C**).

Table 4. JAK2 Mutation Status and Relation to Stage-Specific Expression Levels of MMPs, TIMPs, and COLs

Target gene	Cellular cIMF (n = 31)	Advanced cIMF (n = 33)	Control (n = 24)	Statistical analyses of stage-specific differences (P)	Cellular cIMF +/+ versus +/- versus wt (P =)	Advanced cIMF +/+ versus +/- versus wt (P =)
COL1	0.6 (0.1 to 9.8)	2.4 (0.4 to 41.0)*	1.2 (0.1 to 9.5)	< 0.001*	0.942	0.446
COL3	1.2 (0.3 to 4.3)	5.3 (1.0 to 76.3)*	0.6 (0.01 to 3.3)	< 0.001*	0.222	0.560
MMP-2	1.7 (0.3 to 131.4)	1.5 (0.1 to 7.8)	1.3 (0.3 to 5.7)	ns	0.857	0.327
MMP-8	2.2 (0.3 to 6.0)*	1.2 (0.5 to 11.5)	1.0 (0.3 to 2.8)	< 0.001*	0.119	0.769
MMP-13	0.7 (0.2 to 4.3)	4.6 (0.5 to 83.2)*	1.2 (0.2 to 4.3)	< 0.001*	0.992	0.752
MT1-MMP	0.9 (0.3 to 2.0)	2.4 (0.5 to 7.1)*	0.8 (0.5 to 2.0)	< 0.001*	0.087	0.636
TIMP-1	1.0 (0.2 to 5.3)	1.3 (0.1 to 3.0)	1.0 (0.6 to 1.5)	ns	1.000	0.854
TIMP-2	1.1 (0.3 to 2.3)	1.0 (0.1 to 2.8)	1.0 (0.6 to 1.5)	ns	0.003*	0.857
						(+/- versus wt)

Summary of target genes and expression level (median + range) in cellular cIMF, advanced cIMF, and control group. Significant overexpression of a target gene is displayed in bold. ns, not significant.

*Statistically significant differences in gene expression. Expression of a given target gene was also compared between V617F homozygous (+/+), V617F heterozygous (+/-), and wild-type (wt) JAK2 cases in cellular and advanced cIMF.

Figure 2. COL1, COL3, MMP-13, and MMP-14 showed significantly increased expression in advanced cIMF (**A**, **B**, **E**, and **F**, respectively). MMP-8 mRNA was exclusively up-regulated in prefibrotic cIMF compared to normal hematopoiesis but showed no differences to advanced cIMF (**D**). Apart from single outliers, no differences could be demonstrated in gene expression of MMP-2 and the inhibitors TIMP-1 and TIMP-2 (**C**, **G**, and **H**, respectively). Note that single exaggerated target gene expression levels were not included in the point plots (**A**: one case with advanced cIMF showing a 41-fold increase of COL1; **B**: two advanced cIMF cases with a 25.1-fold and 76.3-fold overexpression of COL3; **C**: two outliers with cellular cIMF showing 41.4-fold and 131.4-fold overexpression of MMP-2; **E**: four cases with advanced cIMF showing a 28.7-fold, 32.6-fold, 34.2-fold, and 83.2-fold increase of MMP-13 expression).

$n = 33$) compared to cellular, prefibrotic cIMF (median, 1.2; range, 0.3 to 4.3; $P < 0.001$; $n = 31$) and normal hematopoiesis (median, 0.6; range, 0.01 to 3.3; $P < 0.001$; $n = 24$). The latter did not differ from each other (Figure 1B). COL-1 gene expression in advanced cIMF ($n = 33$) was increased by up to 41-fold (median, 2.4; range, 0.4 to 41.0) as compared to the prefibrotic phase (median, 0.6; range, 0.1 to 9.8; $P < 0.001$; $n = 31$) and normal hematopoiesis (median, 1.2; range, 0.1 to 9.5; $P = 0.03$; $n = 24$) (Figure 2A).

MMP-2, TIMP-1, and TIMP-2 mRNA Are Constitutively Expressed in cIMF and Normal Hematopoiesis

Prefibrotic cIMF (median, 1.7; range, 0.3 to 131.4; $n = 21$), advanced cIMF (median, 1.5; range, 0.1 to 7.8; $n = 15$), and control hematopoiesis (median, 1.3; range 0.3 to 5.7; $n = 21$) did not differ significantly from each other with regard to expression of MMP-2 mRNA (Figure 1C). TIMP-1 mRNA was also constitutively expressed to similar levels by bone marrow cells in cellular, prefibrotic cIMF (median, 1.0; range, 0.2 to 5.3; $n = 21$), advanced cIMF (median, 1.9; range, 0.8 to 5; $n = 15$), and control hematopoiesis (median, 1.3; range, 0.1 to 3.0; $n = 21$) without notable differences (Figure 2G). TIMP-2 mRNA in prefibrotic cIMF (median 1.1; range 0.3 to 2.3; $n = 13$), advanced cIMF (median, 1.0; range, 0.1 to 2.8; $n = 14$), and normal hematopoiesis (median, 1.0; range, 0.6 to 1.5; $n = 10$) also did not differ from each other (Figure 2H). For a comprehensive illustration of the entire spectrum of gene expression level, see Table 4.

Dynamics of MMP, TIMP, and COL mRNA Expression during the Course of Disease in Progressive Myelofibrosis and Stable Prefibrotic Stages

At presentation of case 1, histopathological evaluation of the bone marrow revealed megakaryocytic and granulocytic proliferation suspicious for a prefibrotic cIMF (mf 0). A control biopsy taken 5 months later revealed prefibrotic cIMF without notable fiber deposition (mf 0). Consecutive sequential trephine biopsies 1 year (mf 2) and 3 years later (mf 3) showed progressive myelofibrosis. The onset of demonstrable fiber deposition was paralleled by increased expression of MMP-14, MMP-13, and COL-3 genes. The manifest myelofibrosis 3 years after initial diagnosis exhibited decreased TIMP-1 and MMP-8 levels (Figure 3).

In case 2 the prefibrotic stage persisted (Figure 4) and was reflected by constantly increased MMP-2 and increased MMP-13. In contrast to progressive myelofibrosis, no change in the expression of MMP-14 was demonstrable. A definite decline in the expression of COL-1 during the course of disease was paralleled by constant TIMP expression. Of note, no notable histopathological change was demonstrable in the sequential biopsies at the evaluation of time points. In these two exemplary courses of disease, no correlation between development

of myelofibrosis and occurrence of JAK2 mutation could be demonstrated. The wild-type JAK2 case showed progressive myelofibrosis (Figure 3) whereas course 2 showed transition from the heterozygous V617F JAK2 status to homozygosity during the follow-up (Figure 4).

MMP-14 Protein Is Predominantly Localized in Endothelial Cells and Megakaryocytes

An intense staining could be observed in advanced stages of cIMF with stronger labeling of megakaryocytes, granulocytic precursors, and endothelial lining of sinusoids (Figure 5A). In normal and reactive bone marrow, MMP-14 (MT1-MMP) immunohistochemistry revealed a weak and inconstant decoration of megakaryocytes (Figure 5B).

MMP-1 Is Rarely Detectable in Bone Marrow Cells Derived from Normal Hematopoiesis and cIMF

Two different primer systems for amplification of MMP-1 (Table 2) in bone marrow cells were tested. The overall detection rate in a qualitative RT-PCR assay was 30% in both cIMF ($n = 12$) and normal hematopoiesis ($n = 12$). MMP-1 RT-PCR amplicons derived from cIMF and normal hematopoiesis were almost undetectable as demonstrated by comparison with MMP-1 amplicons derived from a fibroblast cell line (positive control; data not shown).

Discussion

Until now investigations on the role of MMPs and collagen deposition in cIMF have been hampered due to punctio sicca (dry tap) particularly in advanced stages. An imbalance of increased collagen synthesis and decreased proteolytic action in cIMF seems to be a plausible underlying mechanism. Previous studies investigated plasma of patients with cIMF and related Philadelphia chromosome-negative chronic myeloproliferative disorders for aberrant MMP and TIMP protein levels and suggested that elevated TIMP together with decreased MMP levels might be essential for fibrosis formation.^{27,28} Data on serum markers of collagen metabolism and expression of members of the urokinase-type plasminogen activator system (uPA) suggested an impact on remodeling processes during myelofibrosis.²⁹ A more recent study investigated potential mechanisms of enhanced CD34⁺ cell trafficking in cIMF and demonstrated significantly increased plasma and cellular levels of neutrophil elastase (NE) and MMP-9 along with soluble vascular adhesion molecule-1 (sVCAM-1). However, mononuclear cells from cIMF patients in this study served as a surrogate because bone marrow cells could not be harvested because of manifest myelofibrosis.¹⁴

We took advantage of recent progress in molecular analysis of FFPE trephine biopsies³⁰ and investigated total bone marrow cells of prefibrotic and advanced cIMF

Figure 3. In progressive myelofibrosis, MMP-14 mRNA expression notably increased at the onset of demonstrable fiber deposition along with COL3 and MMP-13. The stage of manifest fibrosis showed decreased TIMP-1 and MMP-8 levels whereas considerable fluctuations in mRNA expression were demonstrable for MMP-2 and MMP-13. Note that the expression level of a given target gene under investigation at initial diagnosis was set to 1. Representative silver impregnations (Gomori) of bone marrow sections revealed progressive myelofibrosis starting in June 2001. This course of disease showed wild-type JAK2 at any time point of investigation. Note that expression level in the first biopsy was set to 1.

for expression of collagens and a broad spectrum of MMPs that preferably degrade collagens. Because the JAK-STAT pathway has been demonstrated to be involved in the regulation of MMP genes and TIMPs,^{15–17} we further tested the hypothesis that cIMF cases showing the V617F JAK2 mutation might differ from wild-type JAK2 cases.

In addition to this, expression of the entire spectrum of MMPs and COLs under investigation showed no correlation to the absence or evidence of an underlying V617F JAK2 mutation in a given case. In particular, the state of zygosity in V617F JAK2 mutated cases in both cIMF groups had no effect on target gene expression. TIMP-2, but not TIMP-1, showed a rather higher expression in cellular cIMF with wild-type JAK2 compared to V617F JAK2 in this group but not in comparison to advanced cIMF and control cases. Not unexpectedly, COL-1 and -3 were increased in advanced cIMF, reflecting the ongoing process of collagen synthesis at this stage. Interestingly, in both prefibrotic and advanced cIMF, no MMP examined here showed a decreased expression compared to normal hematopoiesis. Most notably, MMP-14 and MMP-13 could be demonstrated to be significantly in-

creased in advanced cIMF stages. MMP-14 exhibits various important properties including direct cleavage of collagen fibers, activation of other MMPs (such as MMP-13), and induction of angiogenesis.³¹ In contrast to previous studies that propagated the interaction of multiple MMPs such as MMP-2/MMP-9 and integrins for sufficient angiogenesis,^{32,33} more recent data have demonstrated the essential role for MMP-14 in this process.³⁴ Tissues from MMP-14 knockout mice failed to develop vessels in collagen matrices whereas tissues from mice knocked out for MMP-2, MMP-9, and CD44 generated normal vessels. Increased angiogenesis is a hallmark in the bone marrow architecture of advanced cIMF as demonstrated by increased microvessel density or labeling of endothelial osteoprotegerin.^{35,36} Besides megakaryocytes and myeloid precursor cells, we probably unmasked endothelial cells in advanced cIMF as a major source for MMP-14, suggesting its impact on proliferating vessels in the disease course (Figure 5). Because hematopoietic and endothelial progenitor cells are regularly increased in the peripheral blood of patients with cIMF,³⁷ MMP-14 might play a central role in abnormal cell trafficking. This hypothesis is supported by the conclusion of

Figure 4. The persistence of the prefibrotic stage in a disease course was reflected by persistently increasing MMP-2 and MMP-13 levels restricted to the early phase. In contrast to progressive myelofibrosis, no change in the expression of MT1-MMP was demonstrable. A definite decline in the expression of COL1 could be shown in the disease course paralleled by no change in TIMP expression. Silver impregnation (Gomori) of sequential bone marrow trephines revealed no notable deposition of either reticulin (COL3) or collagen fibers throughout this course of cellular, prefibrotic cIMF. An initial heterozygous state in the years 2000 and 2001 switched to homozygosity in October 2003. Note that expression level in the first biopsy was set to 1.

a previous study that a cell-bound MMP could be involved in increased mobilization of cIMF progenitors.¹⁴ Moreover, a recent study revealed MMP-14 as the key pericellular collagenolysin in the process of pathological vessel remodeling and angiogenesis.³⁸

The investigation of COLs and MMPs in both disease stages of cIMF represents only a snap-shot in the dynamic process of matrix remodeling. To gain more insight into the intraindividual expression, we selected two disease courses showing either progressive myelofibrosis or

Figure 5. Endothelial cells and megakaryocytes in advanced cIMF were prominently stained for MMP-14 (MT1-MMP) protein. **A:** Labeling of endothelial cells (arrows) was demonstrable in extended sinusoids (arrows) along with staining of clustered megakaryocytes and also immature granulopoiesis. **B:** Normal hematopoiesis showed heterogeneously labeled megakaryocytes and also occasionally granulocytic precursors. Original magnifications: $\times 400$ (A); $\times 100$ (B).

persistence of the prefibrotic stage in a comparable time frame. Clear differences in the expression of MMPs (MMP-14, MMP-13, MMP-2) and both COLs were demonstrable between the two courses. Different dynamics in gene expression and the missing correlation of JAK2 status and fibrosis were evident, but investigation of a larger series of sequential biopsies in the course of cIMF is needed to prove reproducibility. Accordingly, these figures per se do not allow us to decide on a course in a prospective manner. In general, it is difficult to illustrate the dynamics of ECM remodeling appropriately because activation of latent MMPs is required but not sufficiently demonstrable, neither by gene expression nor by immunohistochemistry. However, as demonstrated not only for single-case analysis but also in the two courses, the increased MMP-14 levels in progressive myelofibrosis shed light on a probably important candidate in this process.

There is no doubt that MMPs and TIMPs represent only two important systems^{14,27–29} in the complex network of proteolytic enzymes and specific inhibitors. Other factors such as the uPA/plasminogen/plasmin/PAI system, elastases, or stromelysins concomitantly interact in the process of ECM remodeling in cIMF. In addition, deposition of matrix components such as collagens in cIMF is boosted by cytokine action such as interleukins thereby further complicating the network of aberrant mechanisms.

Of note, the presence of the JAK2 (V617F) mutation in all cases under investigation did not correlate with a case-specific fibrogenic potential. In a larger series of sequential biopsies in cIMF not yet investigated for targets involved in ECM remodeling, progressive myelofibrosis was also demonstrable in JAK2 wild-type cases (such as no. 1, Figure 3) or longer lasting prefibrotic stages (such as no. 2, Figure 4) showing JAK2 mutation at initial diagnosis (not published). Therefore, constitutive catalytic activity mediated by mutated JAK2 does not seem to be required for any fibrogenic signals.

We conclude that ECM remodeling in disease stages of cIMF is reflected by aberrant expression of defined collagenase subsets independent of an underlying V617F JAK2 mutation.

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