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/H11549 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 thrombocytopenia, and chronic idiopathic myelofibrosis (cIMF) along with more rare entities such as chronic neutrophilic leukemia and chronic eosinophilic leukemia/hypereosinophilic syndrome.¹ cIMF shows progressive 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.¹⁴ 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

Supported by the Deutsche Krebshilfe (Dr. Mildred Scheel Stiftung 10-2191 to O.B. and H.K.).

Accepted for publication April 27, 2006.

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angiogenesis as hallmarks in advanced cIMF stages require local matrix turnover, i.e., proteolytic action, for full establishment. It has been demonstrated that the JAK-STAT pathway is involved in the activation of genes responsible for synthesis and secretion of MMPs. 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. 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, prebiotic 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. For a summary of patient clinical data, see Table 1.

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

As we previously described, total RNA was extracted from total FFPE bone marrow cells after guanidinium isothiocyanate/protease K-based digestion and conventional organic extraction using phenol/chloroform. Total RNA (1 μg), pretreated with RNase-free (RNase−) DNase (1 U/μg RNA, RO1; 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 manufacturer'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, 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 ΔΔCT method as described.

Briefly, the relative transcript level was expressed as the difference of the Ct− values: C_{C1} [target gene, control group] − C_{C1} [housekeeping gene, control group] = ΔC_{C1} [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 [ΔC_{C1} (control group)]. The target gene expression in different disease stages of cIMF was calculated accordingly as described above: C_{C2} [target gene, cIMF case no. 1, 2, 3, . . . ] − C_{C2} [housekeeping gene, cIMF case no. 1, 2, 3, . . . ] = ΔC_{C2} (cIMF case no. 1, 2, 3, . . . ). To determine the case-specific gene expression in cIMF, every single case of a disease stage, i.e., prebiotic cIMF versus advanced cIMF, was calculated relative to the mean ΔC_{C1} [control group] as follows: fold change = 19 − ΔΔC_{C2}, where ΔΔC_{C2} stands for: ΔC_{C2} (cIMF case no. 1, 2, 3, . . . ) − ΔC_{C2} [control group]. For example: ΔΔC2 = −3.5 means that this case expressed 9.5-fold more transcripts (19−3.5) compared to the control group. For a comprehensive review of the equations underlying this quantification algorithm, see Livak and Schmittgen.

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

<table>
<thead>
<tr>
<th>Primer and probes</th>
<th>Sequence</th>
<th>Reference/product</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1 forward</td>
<td>5'-AAACCTTCCTCCACGCTGTC-3'</td>
<td>GenBank NM 000088, 90 bp</td>
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<tr>
<td>COL1 reverse</td>
<td>5'-CGAGGACGACAGGACC-3'</td>
<td>GenBank NM 000090, 80 bp</td>
</tr>
<tr>
<td>COL1 probe</td>
<td>5'-FAM-TATGGCTATGGATGAAATCAAAAACGGAGAAATT-TAMRA-3'</td>
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<tr>
<td>COL3 forward</td>
<td>5'-CAGGATACCAAGGCCACCTC-3'</td>
<td>GenBank NM 000242, 97 bp</td>
</tr>
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<td>COL3 reverse</td>
<td>5'-TGGACCTTAAGACAGGAGGAAG-3'</td>
<td>GenBank NM 000242, 85 bp</td>
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<td>MMP-1 forward</td>
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</tr>
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<td>GenBank NM 000355, 79 bp</td>
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<tr>
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<td>GenBank NM 000181, 81 bp</td>
</tr>
<tr>
<td>TIMP-1 forward</td>
<td>5'-ACTGTTGGCTGTGAGGATGC-3'</td>
<td>GenBank NM 000242, 97 bp</td>
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<td>TIMP-2 forward</td>
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<td>TIMP-2 reverse</td>
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<tr>
<td>TIMP-3 forward</td>
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<td>TIMP-3 reverse</td>
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<td>5'-AGCGATATTCTGGTGATGAGTGGG-3'</td>
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</tr>
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</table>

*Custom made.

JAK2 as described. We adopted this methodology for analysis of FFPE bone marrow cells. 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 7700 (Applied Biosystems, Weiterstadt, Germany) using 2.5 mmol/L magnesium chloride and 25 ng of DNA. Primers (Applied Biosystems, Weiterstadt, Germany) were: JAK2 forward 5′-TGAGGTATGGCTATGGATGAAATCAAAAACGGAGAAATT-TAMRA-3' and JAK2 reverse 5′-AGAAGAAGACCTCCAGTTTGCAAGATGAGTGGG-3' for JAK2 amplification covering the hotspot mutation site. JAK2 genotyping was performed with an additional 5′-biotin tag in a final volume of 50 μL. Forty-five μL of PCR product was 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, Upsala, 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 μl of annealing buffer (Biotage) and 500 nmoL 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 (~2 μm) were stained with the Ready-to-use (Vector Laboratories). Vectastain ABC kit and the peroxidase component for 30 minutes each. DAB staining was 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.

**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.

**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

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

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 sinuoids (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. 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. More recent studies 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. We took advantage of recent progress in molecular analysis of FFPE trephine biopsies and investigated total bone marrow cells of prefibrotic and advanced cIMF.
for expression of collagens and a broad spectrum of
MMPs that preferably degrade collagens. Because the
JAK-STAT pathway has been demonstrated to be in-
volved 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 correla-
tion 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 exam-
ined 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 vari-
ous important properties including direct cleavage of
collagen fibers, activation of other MMPs (such as MMP-
13), and induction of angiogenesis.31 In contrast to pre-
vious 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 demon-
strated by increased microvessel density or labeling of
endothelial osteoprotegerin.35,36 Besides megakaryo-
cytes and myeloid precursor cells, we probably un-
masked 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 hema-
topoietic 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 traf-
ficking. This hypothesis is supported by the conclusion of

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 2004. 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.
a previous study that a cell-bound MMP could be involved in increased mobilization of cIMF progenitors. Moreover, a recent study revealed MMP-14 as the key pericellular collagenolysin in the process of pathological vessel remodeling and angiogenesis.

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.

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: ×400 (A), ×100 (B).
V617F JAK2 mutation. collagenase subsets independent of an underlying of cIMF is reflected by aberrant expression of defined stages (such as no. 2, Figure 4) showing JAK2 mutation (such as no. 1, Figure 3) or longer lasting prefibrotic 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 immuno-histochemistry. 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 systems14,27–29 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.

Acknowledgments

We thank Ms. Christina Koop and Ms. Sabine Schroeter for their skilled technical assistance; and Dr. Masary Monazahan, Niedersächsisches Landesgesundheitsamt, Hannover, Germany, for the opportunity to perform the pyrosequencing assay on the PSQ 96MA instrument.

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