Stem Cell
INVESTIGATION

Quantitative analyses of myelofibrosis by determining hydroxyproline

Wanke Zhao, Wan-Ting Tina Ho, Zhizhuang Joe Zhao

Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA
Correspondence to: Zhizhuang Joe Zhao. Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA. Email: Joe-zhao@ouhsc.edu.

Background: Myeloproliferative neoplasms (MPNs) are blood malignancies manifested in increased production of red blood cells, white blood cells, and/or platelets. Myelofibrosis is a subtype of MPNs characterized by the formation of scar-like tissues in the bone marrow due to abnormal hematopoiesis. It is considered a disease of both hematopoietic stem cells and stem cell niches. Patients with myelofibrosis have very poor prognosis, and there is no effective treatment so far. Myelofibrosis has routinely been detected by using histochemical staining methods which produce qualitative rather than quantitative results. In this study, we developed a quantitative assay of bone marrow myelofibrosis in JAK2V617F transgenic mice by determining hydroxyproline.

Methods: The JAK2V617F transgenic mice's tissue was collected to detect the bone marrow myelofibrosis. Statistical analyses were performed using the GraphPad Prism program. Differences of samples between two groups were accessed using t tests. P values less than 0.05 (2-tailed) were considered significantly different.

Results: We developed a quantitative method for detecting myelofibrosis by analyzing the content of hydroxyproline, a modified amino acid largely restricted to collagen which forms the fibrotic structure in bone marrow tissues. Our study also demonstrated age-dependent development of bone marrow myelofibrosis in JAK2V617F transgenic mice.

Conclusions: In the present study, we have developed a new method for detecting bone marrow myelofibrosis by analyzing hydroxyproline contents. The method is highly sensitive and accurate. It provides more accurate, representative, and quantitative information than histochemical analyses. We believe that this method should find wide applications for analyzing the progression of myelofibrosis and efficacy of drug treatment.

Keywords: Myelofibrosis; hydroxyproline; method

Background

Myelofibrosis is the most serious form of myeloproliferative neoplasms (MPNs) (1-3). It results from the formation of scar-like tissues in the bone marrow which disrupts normal production of blood cells. The two other forms of MPNs are polycythemia vera (PV) and essential thrombocythemia (ET). PV is characterized by increased production of red blood cells, platelets, and white blood cells, whereas ET is manifest in the elevation of platelets only. Myelofibrosis can

be presented as a primary condition which is named primary myelofibrosis (PMF). It can also be derived from PV and ET which are called post-PV or post-ET myelofibrosis. Patients with myelofibrosis have a very poor prognosis. So far, there is no effective treatment. JAK2V617F, a mutant form of tyrosine kinase JAK2, represents a major molecular defect in MPNs (4-8). It is found in over 95% of PV and over 55% of ET and PMF cases. Transgenic expression or knock-in of JAK2V617F in mice causes ET, PV, and myelofibrosis (9-15). Myelofibrosis is caused by

clonal expansion of malignant hematopoietic cells which have profound impact on their stromal environment. Therefore, myelofibrosis is considered as a disease of both hematopoietic stem cells and stem cell niches (16).

Myelofibrosis is generally detected by staining of tissue sections with silver or trichrome (17). Since collagen types I and III are major components of fibrosis, immunohistochemistry for these two proteins is also commonly used. These staining methods give qualitative data, and subsequent scoring is rather subjective.

Hydroxyproline (4-hydroxyproline) is a non-proteinogenic amino acid formed by posttranslational hydroxylation of proline (18). It is a major component of collagen and has an important role in stabilizing the helical structure. Because hydroxyproline is largely restricted to collagen, the level of hydroxyproline can be considered as an indicator of collagen content. In fact, the hydroxyproline content was found to be correlated with bone marrow fibrosis, and hydroxyproline in urine and serum has been used as a marker to monitor conditions that increase collagen turnover and bone resorption (19-22). In this study, we developed a quantitative assay of bone marrow myelofibrosis in JAK2V617F transgenic mice by determining hydroxyproline.

Materials and methods

Materials

Richard-Allan Scientific Decalcifying Solution which contains EDTA and dilutes hydrochloric acid was purchased from Thermo Scientific. Reticulum Stain Kit and all other chemical reagents were from Sigma-Aldrich.

Mice

JAK2V617F transgenic mice were generated by expressing human JAK2V617F in the hematopoietic system under the control of the *vav-1* promoter (9). These mice have been crossed with wild type C57BL/6 mice for over 10 generations. Homozygous line A JAK2V617F mice were used in this study as previously described (23-25). Wild type C57BL/6 mice were used as control. Animals were housed in ventilated cages under standard conditions. This study was carried out under an approved protocol in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

### Mouse tissue collection and histochemical staining

Mice were put under deep anesthesia through inhalation of isoflurane and terminated by terminal blood collection and open heart surgery. Femurs were dissected and fixed with 10% neutral buffered formalin overnight at room temperature. Following decalcification over night at room temperature by using the Richard-Allan Scientific Decalcifying Solution, the tissue was embedded in paraffin, and 5 μm tissue sections were cut for histochemical staining. Reticulin staining was carried out by using a kit from Sigma-Aldrich following a standard protocol (9,24). Slides were viewed with an Olympus BX-51 upright microscope equipped with U Plan Fluorite objectives. Images were acquired using a DP71 digital camera and processed with the Adobe Photoshop software. Quantification of histochemical staining images was done by using the NIH ImageJ program.

### Acid hydrolysis and hydroxyproline assay

Following formalin fixation and EDTA decalcification, bone marrow tissues were dissected from the bone under a stereo microscope. Bone marrow tissues (usually 5-8 mg, wet weight, from a whole femur) were collected and hydrolyzed with 0.3 mL of 6 M HCl in O'-ring screw-capped tubes at 120 ℃ on a heating block for 3 h. Total protein in the bone marrow was determined by measuring OD_{280nm} and OD_{260nm} of the hydrolysates and calculated based on the Warburg and Christian formula (protein concentration at mg/mL $=1.55 \times OD_{280nm} -0.76 \times OD_{260nm}$). Hydroxyproline assays were performed according to an established protocol (26,27). In brief, the bone marrow hydrolysates were diluted 10-fold in water, and 1-10 μL diluted samples were taken and dried in a speed vacuum concentrator. After the oxidation with chloramine T at room temperature for 5 min, Ehrlich's reagent (p-dimethylaminobenzaldehyde dissolved in n-propanol/perchloric acid) was added and further incubated for 90 min at 60 ℃. The absorbance of reddish purple complex was measured at 560 nm using a spectrophotometer. Standard hydroxyproline was analyzed in the same way, and hydroxyproline contents in the samples were calculated based on standard curves and normalized against total protein concentrations.

Statistical analysis

Statistical analyses were performed using the GraphPad
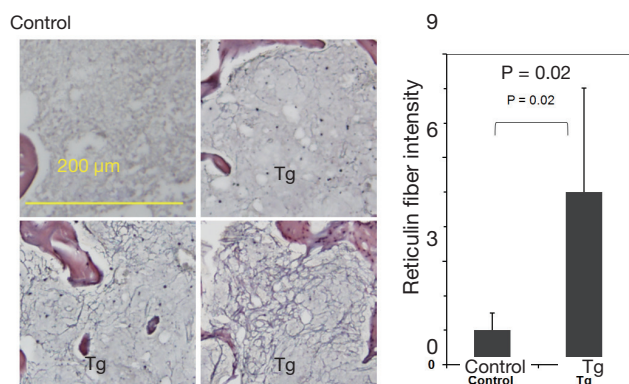
Figure 1 Detection of bone marrow myelofibrosis in JAK2V617F transgenic mice by reticulin staining. Representative reticulin stains of femur sections from a control mouse and a JAK2V617F transgenic mouse (both at 40 weeks of age) are shown. Data in the bar graph represent quantitative assessment of reticulin staining intensity (in arbitrary unit) of randomly selected sections/areas by using the ImageJ software. Error bars denote SD (n=5). The scale bar is applicable to the entire figure.
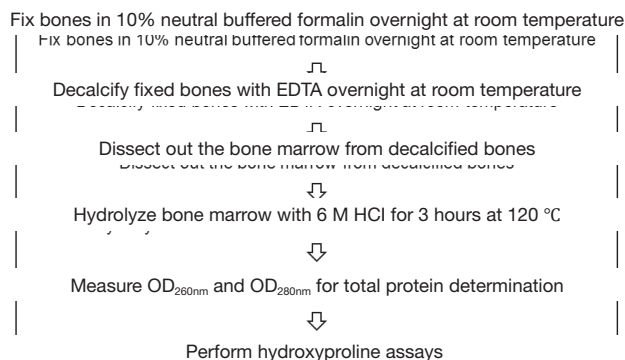
Figure 2 Flowchart for determination of hydroxyproline contents in the bone marrow.

Prism program. Differences of samples between two groups were accessed using *t* tests. P values less than 0.05 (2-tailed) were considered significantly different.

Results

Development of an assay for detection of myelofibrosis by analyzing hydroxyproline in the bone marrow

As reported in our earlier studies, JAK2V617F transgenic mice developed bone marrow fibrosis (9). *Figure 1* shows typical reticulin staining results of bone marrow sections obtained from 40-week-old control and transgenic mice. While reticulin fibers were clearly seen in JAK2V617F transgenic mice, they were essentially absent in the control mice. However, the fibers were not uniformly distributed in the bone marrow of transgenic mice. The intensity of reticulin staining varied substantially in different sections and areas. Although analyses of multiple images by using the ImageJ computer software provided semi-quantitative data, the error is usually large. Therefore, reticulin staining is mainly a qualitative assay and may not be suitable for assessing incremental progression of myelofibrosis in transgenic mice.

Since collagen is the major component of reticulin fibers and hydroxyproline is a largely restricted to collagen, we thought to develop a method to detect bone marrow myelofibrosis by analyzing hydroxyproline contents. A flowchart of the method is shown in *Figure 2*. The procedure contains six simple steps. The two initial steps can be conducted in conjunction with histochemical analyses and were found to be essential for collection of clean and representative bone marrow tissues. The remaining steps follow the standard protocol for chemical analysis of hydroxyproline in proteins. The hydroxyproline analysis results obtained with bone marrow from same mice described in *Figure 1* are shown in *Figure 3*. The results revealed sharp contrast between control and transgenic mice, and the data was representative of a whole femur with a very negligible margin of error. The assay is apparently highly sensitive because a bone marrow sample corresponding to 1/600 of total materials in a mouse femur is sufficient to produce a readout shown in *Figure 3A*. We further demonstrated the linearity of the assay by using different volumes of bone marrow hydrolysates and standard hydroxyproline (*Figure 3B*). The data suggest that the method is able to cover a large range for hydroxyproline assays in the bone marrow.

Revelation of age-dependent development of myelofibrosis in JAK2V617F transgenic mice

We then employed the hydroxyproline detection method to analyze bone marrow myelofibrosis in JAK2V617F mice of different ages ranging from 20 to 60 weeks. Hydroxyproline levels were normalized against total protein contents determined by measuring OD_{280nm} and OD_{260nm} of bone marrow hydrolysates. The data shown in *Figure 4* demonstrated a gradual increase in myelofibrosis in the transgenic mice as they aged. In contrast, control mice

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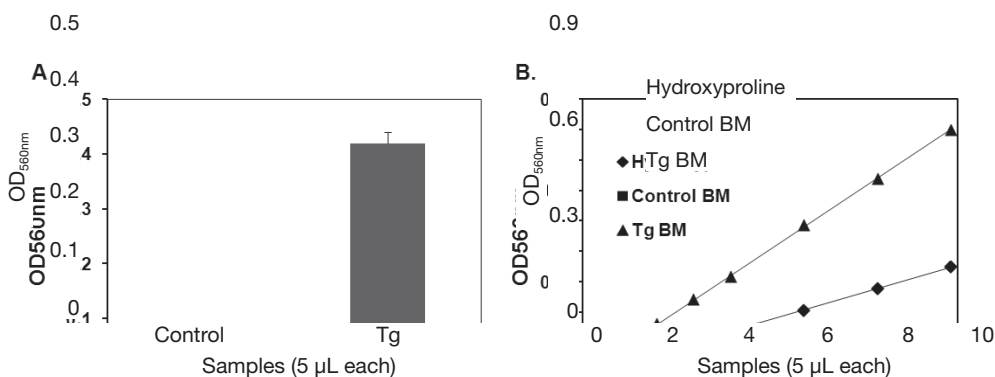
Figure 3 Detection of bone marrow myelofibrosis by analyzing hydroxyproline. Bone marrow tissues from a whole femur of the same 40-week-old control or JAK2V617F transgenic mouse described in *Figure 1* were collected and hydrolyzed with 0.3 mL of 6 M HCl. The hydrolysates were diluted 10-fold in water and then subjected to hydroxyproline analyses in a 0.6 mL assay system. Absorbance at 560 nm was determined by using a spectrophotometer. (A) Assays were performed with 5 μL of diluted hydrolysates from control and transgenic mouse bone marrow samples. Error bars denote standard deviation (n=3); (B) dose response curves of hydroxyproline determination by using indicated volumes of diluted bone marrow hydrolysates or standard hydroxyproline (5 μg/mL). Curves were generated by linear regression.
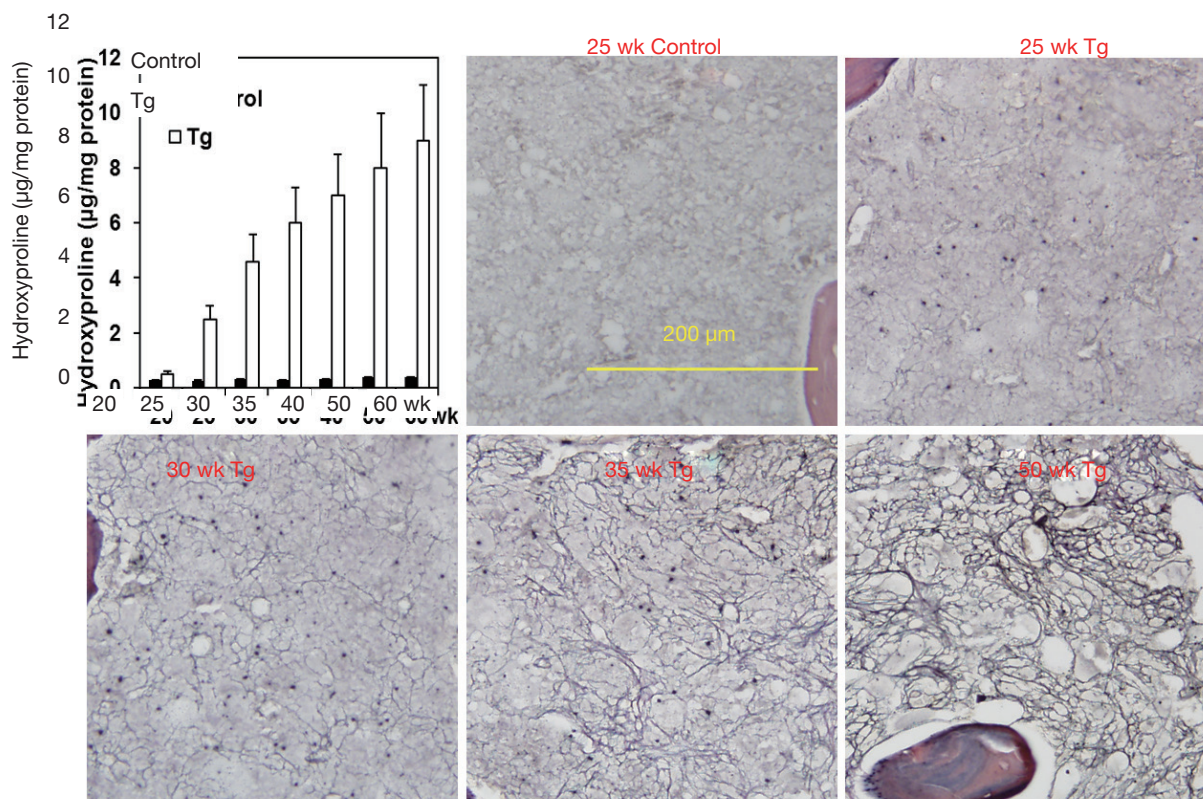
Figure 4 Age-dependent development of bone marrow myelofibrosis in JAK2V617F transgenic mice. Bone marrow samples from control and JAK2V617F transgenic mice of indicated ages (at least 3 mice in each group) were subjected to hydroxyproline analyses as described in Materials and Methods. Hydroxyproline contents are normalized against total proteins in the samples. Error bars denote SD (n≥3). P values are less than 0.01 between control and transgenic mice of same ages. Representative reticulin stains of femur sections from control and JAK2V617F transgenic mice of indicated ages are also shown. The scale bar is applicable to the entire figure.

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displayed a minimal level of hydroxyproline regardless of their ages. Overall, the hydroxyproline analysis data correlated very well with the reticulin staining. However, it should be indicated that at 20 weeks of age, hardly any myelofibrosis was seen with reticulin staining (not shown) but hydroxyproline assay detected a significant difference between control and transgenic mice. Together, the data provide quantitative data demonstrating age-dependent development of myelofibrosis in JAK2V167F transgenic mice. We thus provide a useful tool to detect myelofibrosis at earlier stages and to follow the progression of the disease quantitatively.

Discussion

In the present study, we have developed a new method for detecting bone marrow myelofibrosis by analyzing hydroxyproline contents. The method is highly sensitive and accurate. It provides more accurate, representative, and quantitative information than histochemical analyses. We believe that this method should find wide applications for analyzing the progression of myelofibrosis and efficacy of drug treatment.

For analysis of bone marrow tissues, fixation and decalcification are necessary to collect clean and representative samples. This is because bone marrow cells can't be recovered effectively by simple flushing or cracking especially for those with extensive myelofibrosis. After fixation and decalcification, fixed bone marrow tissues can be easily recovered in a whole piece. This works for small as well as big bones. We have also tried whole bones for direct acid hydrolysis followed by hydroxyproline determination. However, the bone tissue itself and associated cartilage gave rise to a very high background, making the detection inconsistent. Nonetheless, the method can be used to analyze myelofibrosis in the spleen with fresh, frozen, or formalin-fixed tissues. Despite a significant background caused by abundant vascular structures, JAK2V617F-induced spleen myelofibrosis is clearly reflected by an increase in hydroxyproline contents (data not shown). It should be pointed out that formalin fixation of tissues does not affect detection of hydroxyproline.

Acknowledgements

Footnote

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