Pericyte coverage of abnormal blood vessels in myelofibrotic bone marrows

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Supplementary information

Patients

Bone marrow biopsies from 19 MF patients (10 men and 9 women; age range, 50 to 84 years; mean age, 66 years) and nine control subjects (8 men and one woman, 48 to 85 years; mean age 66 years),were retrieved as part of a routine diagnostic procedure. The patients were diagnosed and followed up at Depts. of Haematology, Karolinska University Hospital Huddinge, Stockholm, or Odense University Hospital, Denmark. Duration of the disease was 0-3 years. The diagnosis was based on the WHO classification and all cases were established fibrotic MF and not prefibrotic. All subtypes of MF – idiopathic myeloid metaplasia (IMF; 13 patients), postpolycythemic myeloid metaplasia (PPMF; 4), and postthrombocythemic myeloid metaplasia (PTMF; 2) - were represented. Four patients were previously treated, but only 3 were receiving treatment (2 with hydroxyurea and 1 with Epo and G-CSF) at the time of bone marrow sampling. Five IMF patients were positive for the JAK2 V617F mutation, and 5 were negative. For 9 MF patients JAK2 status could not be obtained.

Two patients with secondary MF due to Sjögren's syndrome and large granular lymphocytosis (LGL) associated severe neutropenia were also included in this study. The Sjögren syndrome patient has been reported previously[17] and we used bone marrow samples obtained prior to treatment with intravenous gammaglobulin.

The 9 individuals in the control group underwent biopsy because they had an¬emia or leukocytosis; they were found to have normal bone marrow according to a routine examination by a pathologist and they presented no clinical evidence of a malignant condition on follow-up for several years. All bone marrow samples were paraffin-embedded.

GATA-1[low] mice

GATA-1[low] mice were obtained through the targeted replacement of ≈8kb upstream sequences of the GATA-1 locus, including the distal GATA-1 promoter in a DNase I hypersensitive region, overall resulting in the selective abrogation of GATA-1 expression in cells of the megakaryocytic lineage [18]. The GATA-1^{low} colony was bred according to standard genetic protocols at the animal facilities of the Istituto Superiore di Sanità (Rome, Italy) as described.[15] All the experiments were performed under protocols approved by the institutional animal care committee. Samples of spleen (n=3) and bone marrow (n=2) were fixed in formalin and paraffin embedded; then, 2.5-3 μm thick sections were prepared.

TPO over-expressing mice

Lethally irradiated (10 Gy) wild type (WT) mice (8-10 weeks old) were used as hosts. They were injected via the retro-orbital sinus with 4×10^6 lineage negative (Lin-) cells exposed to the MIGR-TPO virus.[12]

To generate a TGF-β1 free background, heterozygote TGF-β1 breeders with a mixed Sv129 × CF-1 genetic background were used. Since all homozygote TGF-β1$^{-/-}$ animals die before weaning, single cell suspensions were prepared from femurs and tibiae from 14- to 16-day-old homozygous pups. The Lin- fraction was mixed with MIGR-TPO virus and inoculated in lethally irradiated WT hosts 12.

Bone marrows were fixed and paraffin embedded; then 2.5-3 μm sections were prepared. For each genetic manipulation as well as from WT, marrow sections from 2 different mice were investigated.

Antibodies

Primary antibodies, directed against human antigens were: for CD34, a mouse monoclonal IgG1 (Qbend10) from Biogenex, (San Ramon, CA, USA); for SMA-α a FITC conjugated anti SMA-α IgG2a (DAKO A/S, Glostrup, DK), diluted 1:600,; for desmin, a mouse monoclonal IgG1 from DAKO diluted 1:50; for PDGFR-β, a rabbit polyclonal (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA); and for VEGF-A, a rabbit polyclonal from Biogenex. Secondary antibodies were: Alexa 568 and 546 goat anti-mouse IgG1 and Alexa 488 goat anti-mouse IgG2a, all from Molecular Probes Inc (Eugene, OR, USA), diluted 1:800 or as indicated. In the mouse studies, primary antibodies were: a rabbit anti-mouse CD34 from Cedarlane (Hornby, Ontario, CA); a rabbit anti-mouse CD31 from BD Biosciences Pharmingen (Mountain View, CA, USA); a HRP-conjugated and a FITC-conjugated monoclonal mouse anti-SMA-α, and a HRP-conjugated monoclonal mouse anti-desmin, both from DAKO.

Immunofluorescence and immunohistochemistry

Human specimens. To visualize vessels and PC by immunofluorescence, cells were labeled with CD34 or SMA-α antibodies by standard techniques. Marrow sections were deparaffinized with xylene and rehydrated by serial incubations with decreasing concentrations of ethanol.[5] For some studies, we reduced background fluorescence by incubating sections with 0.25% ammonium in 70% ethanol for one hour.[24] Then, sections were blocked with 3% goat serum. Primary antibodies were applied for 60 min at room temperature and, after washings, sections were incubated with secondary antibodies for 30 min.

In double stainings the 2 primary antibodies were added together. To further reduce background staining, some sections were incubated in 0.5 % sudan black in 70% ethanol for 30 min 24. After washings sections were mounted in 80 % glycerol.

We also tried to visualize PC with antibodies directed against PDGFR-β and desmin using the same procedure as described above.

For VEGF immunohistochemistry, sections were prepared similarly but were also blocked with avidin and biotin. A secondary biotinylated goat anti-rabbit antibody was applied (BioGenix) and, after streptavidin-peroxidase complex was added, 3.3'-diaminobezidine (DAB)(BioGenix) was used as substrate. - To ensure accuracy of the method, each stained sample (counterstained with hematoxylin) was reviewed independently by 2 of the authors, in a blinded fashion.

Staining for SMA-α and desmin was performed as above, but epitope demasking was performed by microwave treatment in citrate buffer, pH 6.0. Sections were blocked with 0.1% bovine serum albumin for 30 minutes and primary HRP-conjugated antibody was added. After washings, DAB was added and sections were counterstained and mounted as above.

Mouse specimens. Staining for CD34 and CD31 on paraffin embedded bone marrows or spleens were performed similarly, but to avoid non-specific staining due to mouse antibodies interacting with mouse tissue a biotinylated secondary mouse adsorbed rabbit anti-mouse antibody was used (Vector, Burlingame, CA). To increase the signal we used an enhancement step using biotinyl tyramid (Perkin Elmer TSA Biotin System kit).

Double staining for CD34 and SMA of mouse sections were carried out with the chromophore fast red, which is visible in both in ordinary and fluorescent light. Sections were stained for CD34 essentially as described above, but levamisole was used for blocking and alkaline phosphatase conjugated streptavidin and fast red (StrAviGen Super sensitive kit, Biogenix) was used to visualize positive cells. After the developing reaction, the FITC-conjugated anti SMA-α antibody was added and, subsequently a secondary Alexa 488 anti-mouse antibody.

Morphometric analyses

MVD in human bone marrows was determined by confocal microscopy. Vessels were counted in 63x high power fields (HPF). To obtain a representative number of vessels 5 HPFs were counted. In mice, MVD was assessed as the mean number of stained vessels per 400 x HPF 3, calculating the mean of 5 randomly chosen areas 3. In order to be considered as a vessel, a lumen or a longitudinal structure of more than the diameter of 3 bone marrow cells or with branching was required, together with elongated nuclei compatible with endothelial cells. The presence of erythrocytes within the lumen was also discriminative. Counting was performed by two separate investigators in a blinded fashion.

We used the image analysis system Leica Q5501W with color video camera DM RXA for light microscopy together with a software system for measurements of blood vessel characteristics, developed with the Leica Qwin Image Analysis (*Miriam Mints EZ, Bo Blomgren, Christian Falconer RRJP. Vascular abnormalities in the endometrium of menorrhagia patients. Fertil Sterility 2007; in press*). Ten randomly selected and crosscut vessels per slide were captured with a 63x oil immersion objective and the perimeter was assessed by manually tracing the inner (luminal) CD34 staining.

3-dimensional imaging

30-50 μm thick tissue slabs were attached to Superfrost Plus glass slides (VWR, West Chester, PA, USA), deparaffinized with Neoclear Xylene substitute (VWR), and rehydrated as described. The tissue was blocked with 3% normal goat serum in phosphate buffered saline with 0.05% Triton X 100 (Baker Chemical Co., Phillipsburg NJ, USA). All procedures were carried out at room temperature. Primary antibodies, i.e. mouse anti-CD34 IgG1 and 1:100 mouse SMA-α IgG2a, were incubated for 2 hours. After washing and blocking, tissue was incubated for 1 hour in Alexa 546 IgG1 diluted 1:500 and Alexa 488 IgG2a diluted 1:800 in blocking buffer. Tissue was washed and mounted onto slides in Fluoromount mounting media (Southern Biotech, Birmingham, AL, USA), and scanned under 40x and 100x oil immersion lenses using a Leica TCS NT confocal laser scanning microscope (Leica, Exton, PA, USA) fitted with aircooled argon and krypton lasers adjusted for optimal serial section acquisition.[19] Stacks of serial sections were then rendered in 3 dimensions using VoxelView 2.5.1 software (Vital Images Inc., Fairfield, IA, USA) to examine the 3-dimensional architecture of the endothelial wall constituents. Vessels, selected for 3-D analysis, were identified by the presence of CD34 positive cells as described. Projections sequences were constructed from these data without filtering or segmentation for viewing purposes.

Supplementary figures
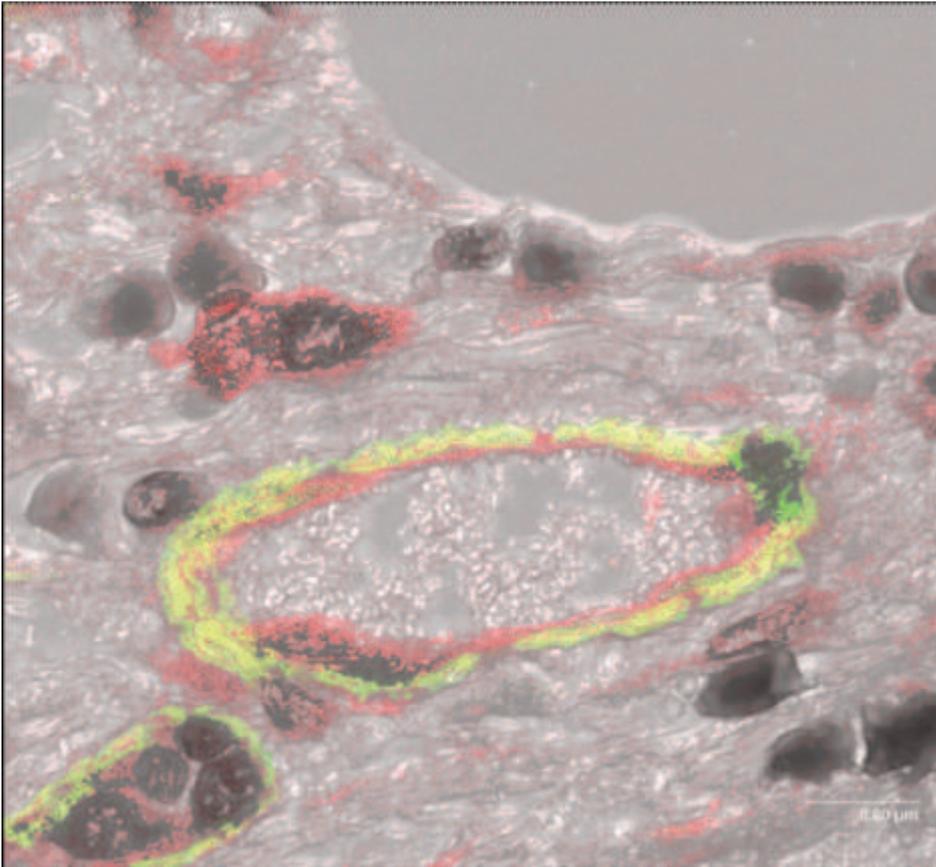
Figure 8. To identify pericytes in myelofibrosis bone marrow, biopsies were double stained for PDGF-Rβ (red) and SMA-α (green). As seen in the overlay picture, a majority of cells forming a vessel-like structure stain positive for both PDGF-Rβ and SMA-α (thus appearing with yellow color), identifying them as pericytes.
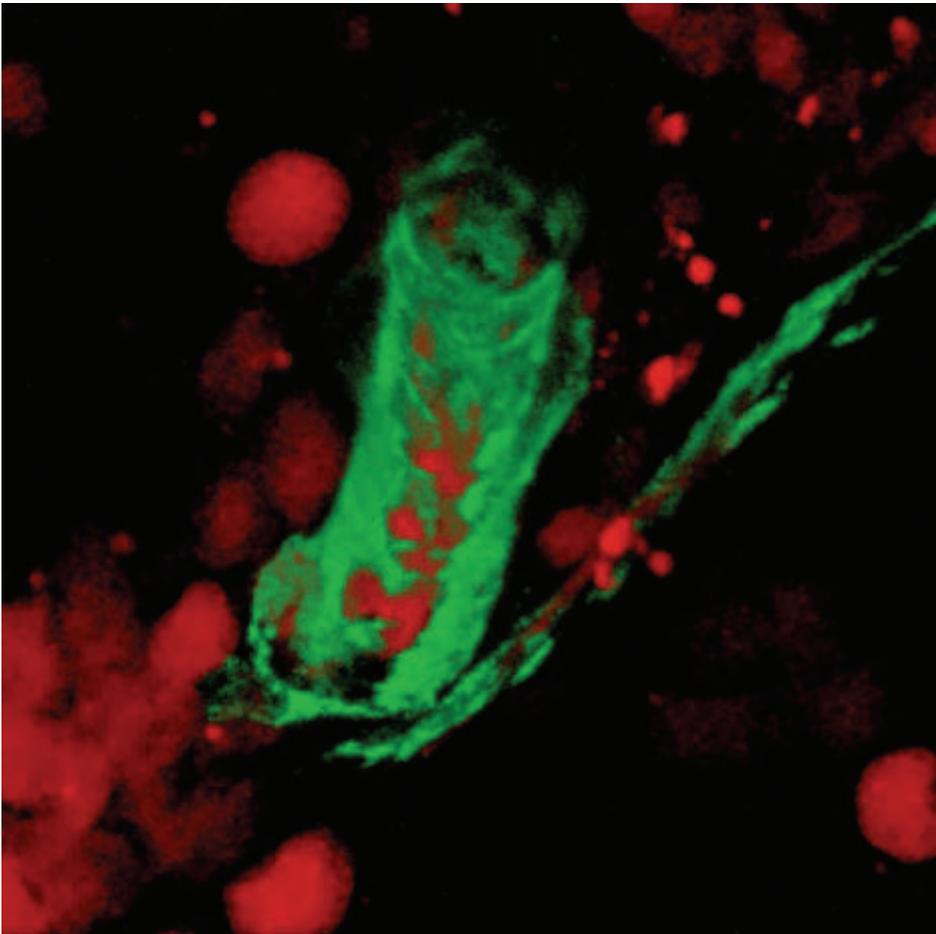
Figure 9. Video recoding shows a 3D-reconstruction of a capillary from the bone marrow of a MF patient. Green cells are pericytes (stained for SMA-a) and red cells are endothelial cells (stained for CD34). Note that part of the vessel appears to consist only of pericytes.