megakaryocyte and platelet lysates should take into consideration that the MMP-2, -3, and -9 activities may be significantly inhibited by calcium chelators and phosphotyrosine phosphatase inhibitors. More cautious use of these MMP inhibitors will provide useful insights to understand how cytoplasm and nuclear MMPs (F.M. and V.M., unpublished observations, October 2011) may be transferred from megakaryocytes to platelets in physio-pathologic conditions.

Figure 1. Gelatin zymograms of cord blood and mature platelets from healthy individuals. Human platelets from peripheral blood and cord blood samples were obtained from the Blood Transfusion Center and the Gynecology Unit of the Hospital of Urbino. Umbilical cord blood samples were collected immediately after delivery from women with uncomplicated healthy pregnancies; peripheral blood samples were drawn from healthy volunteers. Washed platelets were freshly isolated according to CD45 leukocytes depletion and lysis procedure detailed in Cecchetti et al (40 mM Tris-HCl, 0.3M NaCl, 1 mM EDTA, 1 mM Na2VO4, 1 mM NaN3, NaH2PO4 0.05%, NP-40 1%, pH 7.4). After centrifugation (20000 g, 4°C for 20 minutes) to remove cellular debris, the supernatates of MMP standards from whole cord blood and the cleared platelet lysates were analyzed through Western blotting (using monoclonal antibody against MMP-2 and MMP-9) and gelatin zymography. Sample aliquots (containing 250 μg of total protein) were analyzed on 7.5% polyacrylamide gels containing 2% gelatin 90 Bloom Type A from porcine skin. (A) Western blots of pro- and complexed forms of MMP-9, and proMMP-2 (lanes 1 and 2, respectively) recognized as gelatinases circulating in human cord blood from healthy subjects. Lane standard, cord blood lysates used as calibrator. (B) In lane 1, the MMP gelatinolytic activities in mature platelet lysates. All MMP forms activated by 1 mM APMA are separated in lane 2, whereas the residual gelatinolytic activity of APMA-activated MMP in platelet lysates after the treatment with 1 mM EDTA and orthovanadate is shown in lane 3. Lane standard, cord blood gelatinases.

Response

MMP-9 in platelets: maybe, maybe not

We recently demonstrated that megakaryocytes differentially express mRNA for MMPs and TIMPs and selectively transfer a subset of these transcripts to platelets. As a minor part of this report, we showed that megakaryocytes expressed MMP-9 mRNA while platelets contained only trace amounts of it and undetectable MMP-9 protein. In their letter to the editor, Mannello and Medda suggest that our results are inconsistent with previous reports showing MMP-9 zymographic activity in platelet lysates. Although the authors did not show that leukocyte-depleted platelets express MMP-9 protein, they argue that MMP-9 activity is detectable in platelets when EDTA and Na2VO4, that may inhibit MMP-9 activity, are removed from the lysis buffer.

The effects of EDTA on the activity of MMPs detected by zymography in biologic samples are controversial, with data showing partial inhibition, no influence, or enhancement of active MMP-9. In our current study and a previous report, we were unable to detect MMP-9 protein in platelets by ELISA, immunocytochemistry (ICC), and Western blotting (WB). EDTA and Na2VO4 are not used when detecting MMP-9 protein by ICC or WB analysis. Moreover, the presence of EDTA and Na2VO4 does not mask the detection of MMP-9 in ELISA-based assays.

As duly noted in our report, it is possible that MMP-9 protein, if it exists in platelets, escaped the detection limits of our assays. However, we were unable to detect enzymatic activity in platelets using both zymography and a sensitive activity assay, whereas...
MMP-9 activity was readily detected in collagen-adherent monocytes that were costimulated with platelets. To address the influence of possible methodologic artefacts, we have now performed additional experiments in leukocyte-depleted human platelets using 3 different procedures: (1) complete lysis buffer, as detailed in our paper; (2) the same buffer but without EDTA and Na3VO4; and (3) 3 cycles of freezing and thawing. We were unable to detect zymographic MMP-9 activity in any of the preparations (Figure 1A), while the addition of minimal amounts of leukocytes to purified platelets elicited MMP-9 activity (Figure 1B). This finding is consistent with next-generation RNA-sequencing data showing that human neutrophils express far higher amounts of MMP-9 transcripts than platelets (Figure 1C). Therefore, contamination by white blood cells may explain the finding of MMP-9 in platelets reported in previous studies.

We subscribe to the position that strict attention must be paid to methodologic issues when studying the pathophysiology of MMPs, which is why we screen MMPs by multiple assays that include in situ detection of the protein within individual platelets and broader assessment of mRNA, protein, and activity using platelet preparations devoid of leukocytes. Using these criteria, we were unable to detect appreciable amounts of MMP-9 in platelets. Similarly, Kälvegren et al recently demonstrated that collagen-stimulated platelets release MMP-1 and MMP-2, but not MMP-9. Moreover, they were unable to detect MMP-9 protein inside resting platelets by immunofluorescence microscopy. Thus, it is not obvious to us that platelets from healthy subjects possess appreciable amounts of MMP-9. Nevertheless, we encourage further scientific dialogue and evaluation of this issue as our group and others determine how platelet-derived MMPs participate in diseases.

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Figure 1. Detection of activity and mRNA of MMP-9 in platelets. (A) Zymography of highly purified platelet lysates prepared according to 3 different methods (lane 3 = complete lysis buffer; lane 4 = the same lysis buffer but without EDTA and Na3VO4; lane 5 = 3 cycles of freezing in liquid nitrogen and thawing at 37°C). Two concentrations of MMP-9, used as standards, are shown in lanes 1 and 2. No MMP-9 is detectable in platelet lysates prepared by any of the 3 methods. (B) Zymography of highly purified platelets, alone (lane 1) or with added white blood cells at platelet leukocyte ratios of 789:1 (lane 4), 24 000:1 (lane 3), and 47 000:1 (lane 2). PMNs lysate and a concentration of MMP-9 used as standards, are shown in lanes 5 and 6, respectively. (C) RNA-seq analysis for MMP-9 mRNA in platelet and PMN lysates. Matched sequences are aligned to the MMP-9 gene using the Integrated Genome Browser (IGB). Gene map (bottom portion of the panel, oriented 5'-3' direction) is represented by thick (exon) and thin (intron) lines. The transcript levels were quantified using RPKM (reads per kilobase of exon model per million mapped reads). The RPKM for adult PMNs without any treatment is 82.6916 compared with 0.4619 for platelets.
To the editor:

Some observations on the geometry of megakaryocyte mitotic figures: Buckyballs in the bone marrow

In a typical cell, the centrosome replicates at G1-S and then the daughter migrates to the opposite side of the nucleus to help form a biphasic spindle as the cell prepares for division. The position of the centrosomes is critical for ensuring symmetry and segregation of the chromosomes to the progeny. The positioning is also important for cell organization and for asymmetric divisions like in stem cells. It is believed that the centrosomes move along a meshwork of microtubules propelled by pushing or pulling with motors from dyneins or kinesins. But how they “know” when they attempt to orchestrate anaphase. Four equidistant points on a sphere form a tetrahedron. Eight vertices are needed. But what geometry is there for a polygon with 16 vertices?

Megakaryocytes are giant platelet-producing cells in the marrow that develop through endomitosis, skipping cell divisions and increasing chromosome complement from 2N to 4N to 8N, and so on. The mechanism of this polyploidization is not well understood, but must be extraordinarily complex because for an averaged-sized 32N megakaryocyte almost 750 chromosomes have to be organized onto 16 mitotic plates, tethered to a spindle, and carefully separated during what will be a mitotic cycle with an abortive anaphase and cell division.

As a Buckminster Fuller enthusiast from the 1970s, I was excited to identify during a bone marrow biopsy examination a 32N megakaryocyte mitosis with almost perfectly organized mitotic plates resembling a geodesic dome (Figure II). Given the excitement generated by the discovery of the geodesic C60 molecule, appropriately named Buckminsterfullerene, and referred to as a Buckybball (appearing on the cover of Science when it was molecule of the year in 1991), I wondered if these megakaryocyte mitoses were Buckybballs in the bone marrow. Could they shed light on the organization of the mitotic apparatus? I attempted to discern the geometry.

Mitotic figures in megakaryocytes are infrequent and those with separated mitotic plates that allow for an examination of geometric relationships are exquisitely rare. Nevertheless, over the years images of 16N mitoses were collected; surprisingly, they did reveal an underlying symmetry. In the 16N images (eg, Figure 1E), the angles of the mitotic plates formed vertices of triangles, the triangles formed tetrahedra, and the entire configuration appeared to form a stella octangular (Figure 1F). This is an unusual geometric composed of 2 tetrahedra one inverted and rotated 180° with respect to the other. Although perplexing, the double tetrahedron for 16N implied a single tetrahedron for 8N, and this was confirmed with examples (Figure 1A-D). In an 8N cell, it seems the centrosomes appear to localize in the corners of a cube. Eight vertices are needed. But what geometry is there for 32N? The relationships are exquisitely rare. Nevertheless, over the years next perfect geometries are the icosahedron and dodecahedron with 20 and 12 vertices respectively; hardly what is required to continue the polyploidization to 32N. Sixteen vertices are needed. But what geometry is there for a polygon with 16 vertices?

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References
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