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Two-dimensional analysis of the structure of human von Willebrand factor

Paolo Perutelli, Silvia Catellani, Pier Giorgio Mori

Hematology Laboratory, Hematology and Oncology Department, G.Gaslini Children's Hospital, Genova, Italy

Human von Willebrand factor (vWF) is synthesized as an extra large polymer; then, it is converted to lower molecular weight plasma multimers, originally composed of intact 225-kDa subunits, by a metalloproteinase.[1,2] Proteolysis generates two fragments of 140- and 176-kDa, which originate from cleavage of peptide bond Tyr842-Met843 and which represent vWF residues 1-842 and 843-2050, respectively; a very small amount of 189-kDa fragment can also be found in normal plasma.[3] We describe here a two-dimensional (2-D) method to analyze plasma vWF structure.

Agarose gel electrophoresis was used as the first dimension to resolve the multimeric structure of vWF; the second dimension, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), allowed us to obtain information about proteolysis *in vivo* of this molecule. One-dimensional electrophoresis was performed on a vertical mini-gel apparatus (Mini-Protean II, Bio-Rad, Hercules, CA, USA). Plasma from normal subjects and type 2 von Willebrand disease (vWD) patients was diluted 1:5 in sample buffer; 30 µL were applied on 1.7% low gelling temperature agarose and run at 14 V for 20 h.[4]

vWF lanes were excised from the agarose gel, washed in distilled water for 30' and soaked in reducing buffer (1% dithiothreitol and 1% SDS in stacking gel buffer, pH 6.8) for 30' at room temperature under gentle agitation.

SDS-PAGE (5% polyacrylamide)[5] was performed on mini-slab gel. The reduced lanes were applied on the top of the gel and electrophoresis was run at 200 V until the dye front reached the bottom of the gel.

Immunodetection was performed as described elsewhere;[4] briefly, after electroblotting of 1-D gel, nitrocellulose membranes were incubated with rabbit anti-human vWF antiserum followed by incubation with alkaline phosphatase-labeled anti-rabbit antibody.

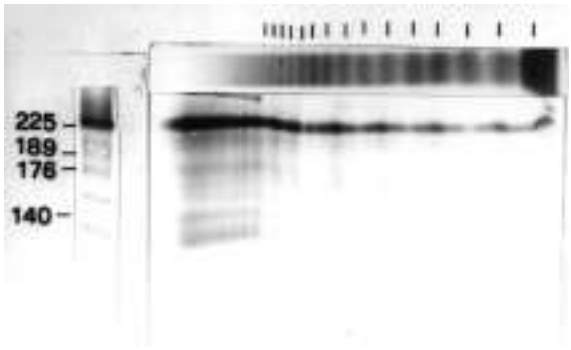

Figure 1. 2-D analysis of plasma normal vWF. Multimeric and subunit composition of vWF are shown, as references, on the top and on the left of the figure, respectively.

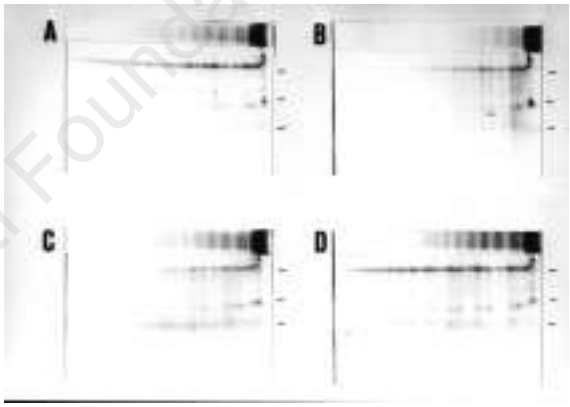

Figure 2. 2-D analysis of type 2A (A,B,C) and type 2B (D) vWF. Dashes indicate 225-, 176-, and 140-kDa subunits, respectively.

BCIP/NBT was used as the chromogenic substrate.

Normal vWF shows a predominance of the intact 225-kDa subunit; the proteolytic fragments are present in low amounts (Figure 1). The 189-kDa fragment is poorly recognizable due to its low concentration in plasma. All subunits are resolved as broad bands having a whiter central area; the high glycosylation degree of vWF[6] may be responsible for this pattern. Moreover, better detection of vWF subunits may be achieved using a pool of monoclonal antibodies rather than a polyclonal antibody.

Type 2A and 2B vWF lack the higher molecular weight multimers;[7] 2-D analysis shows that the lower molecular weight multimers are composed of higher amounts of proteolytic fragment than normal plasma (Figure 2).

The proposed method allows 2-D analysis of vWF; in this way, we could achieve information on multi-

merization and proteolysis of vWF. The method is simple, based on well-tested techniques such as agarose gel electrophoresis, SDS-PAGE, and immunoblotting; it is performed by mini-gel equipment, thus minimizing reagent consumption and analysis time. Moreover, vWF subunits are immunoenzymatically detected, without need of radiolabeled reagents.

Key words

von Willebrand factor, two-dimensional analysis

Correspondence

Paolo Perutelli, PhD, Hematology Laboratory, Hematology and Oncology Department, G. Gaslini Children's Hospital, largo G. Gaslini, 5, 16147 Genoa, Italy. Phone: international +39-010-5636277 •Fax: international +39-010-3776590.

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Long-term disease-free acute promyelocytic leukemia patients really can be cured at molecular level

GIOVANNI MARTINELLI, EMANUELA OTTAVIANI, GIUSEPPE VISANI, NICOLETTA TESTONI, VITTORIO MONTEFUSCO, SANTE TURA

Institute of Hematology and Medical Oncology "Seràgnoli" ; University of Bologna, Italy

The characteristic t(15;17) translocation involving chromosomes 15 and 17 is specifically associated with both the common and the variant subtypes of acute promyelocytic leukemia (APL) (M3 according to FAB classification).[1] At the molecular level, it fuses genes encoding PML on chromosome 15 and the nuclear retinoic acid receptor-α (RARα) on chromosome 17. The subsequent expression of PML/RARα fusion mRNA provides a potential molecular marker that can be detected in leukemic cells taken from patients with APL.[1] Using PML and RARa sequence specific primers, reverse transcription-polymerase chain reaction (RT-PCR) assays have been developed for detection of PML/RARα transcript in leukemic cells obtained from patients; these RT-PCR assays are more sensitive than conventional cytogenetic analysis.[1,2]

We and others reported previously that the majority of the acute promyelocytic leukemia (APL) patients with long-lasting disease free survival were negative for PML/RARα transcript. We have now applied RT-PCR assay for PML/RARα analysis on bone marrow samples from 18 APL patients (8 female, 10 male; median age 31 years; range 14-59) with long-lasting complete remission (CR), after induction chemotherapy and consolidation (median 59 months; range 38-142 months from CR) in order to verify the validity of these observations further. All patients were in clinical and cytogenetic CR at the time of molecular evaluation. Nine of these patients had already been studied.[2] In eleven patients karyotypic analysis on bone marrow aspirates was performed at diagnosis and confirmed the presence of the t(15;17) translocation. In the other 7 patients, using bone marrow samples frozen at the time of diagnosis we were able to detect the presence of the PML/RARα transcript by RT-PCR analysis.[3]

Patients received different protocols of induction chemotherapy including an anthracycline (daunorubicin or idarubicin) alone or in combination with cytosine arabinoside (biological and clinical data are given in Table 1). After achievement of CR, one patient (PS in Table 1) was submitted to allogeneic bone marrow transplantation (BMT) from an HLA matched available donor. Fourteen patients were submitted to autologous BMT.[4] Only two patients (GL and OD) were submitted to maintenance chemotherapy, and one patient (OM) withdrew from maintenance chemotherapy owing to hepatic toxicity. Remission bone marrow aspirates were obtained after achievement of CR and used for molecular analysis.[1] Cytogenetic studies were performed as reported.[3] RT-PCR analysis was performed as described elsewhere.[2] Concerning the specificity and sensitivity of our RT-PCR method, we can detect one PML/RARα-positive cell diluted in 10^{-3}-10^{-4} PML/RAR-negative cells.

The results of RT-PCR analysis in remission samples are schematically represented in Figure 1. Only the molecular results regarding the last sample for each patient are presented. In all cases but one, no PML/RARα transcripts were visible either on the ethidium bromide gels or after silver staining. At present, all but one of the patients are in continuous CR with a median follow up of 59 months (range 38-142). The patient who died (OM) had been persistently PCR positive at different times of analysis (+13, +15 and +32 months). After 39 months of CR, she presented a cytogenetic and a clinical relapse. A second CR was achieved after therapy with all-trans retinoic acid