To the editor:

Bovine protein disulfide isomerase-enhanced tissue factor coagulant function: is phospholipid contaminant in it the real culprit?

Tissue factor (TF) initiates coagulation after the binding of clotting factor VIIa (FVIIa). Only a small fraction of the total TF present on cell surfaces is coagulant-active; the majority is inactive or cryptic. At present it is not completely clear how the TF that is active in coagulation differs from the cryptic form or what mechanisms are involved in TF activation. It has been suggested recently that protein-disulfide isomerase (PDI), through its oxidoreductase activity, regulates TF function on cells. The validity of this hypothesis has been questioned. Interestingly, Versteeg and Ruf more recently reported that PDI, independent of its thiol-oxidoreductase activity, markedly increased TF coagulant activity. In these experiments the enhancing effect of PDI was seen only with soluble TF (sTF) or microparticle TF and used commercially obtained purified bovine PDI (Sigma-Aldrich, St Louis, MO). In contrast to the published report, we saw no significant effect of recombinant human PDI (rhPDI) on TF activity in our preliminary studies. In light of this discrepancy we have reexamined the effect of bovine PDI and rhPDI on sTF activity. The bovine PDI used in the present study is from the same supplier as the bovine PDI used by Versteeg and Ruf. Recombinant hPDI was obtained from 2 different sources, Biovision (Mountain View, CA) and RayBiotech (Nocross, GA). sTF was kindly provided by Wolfram Ruf (Scripps Research Institute, La Jolla, CA) and annexin V was provided by Jonathan Tait (University of Washington, Seattle, WA). In accordance with their report, bovine PDI increased the activity of sTF (Figure 1A) both markedly and in a dose-dependent manner. In contrast to the results seen with bovine PDI, rhPDI failed to increase the activity of sTF in a significant manner (Figure 1A). The dramatic difference between bovine PDI and rhPDI in their ability to enhance the activity of sTF could be a real phenomenon and reflect differences between these molecules or one of the above findings could be artifactual. It is pertinent to note here that sTF has minimal coagulant activity, which can be increased greatly in the presence of anionic phospholipids (PL). If the commercially obtained bovine PDI that was purified from liver (also a starting material for purification of PL in many cases) contained traces of PL, it would markedly increase the activity of sTF or microparticle TF without

Figure 1. Evidence that traces of anionic phospholipids present in commercially obtained bovine PDI is responsible for increasing sTF activity. (A) Bovine PDI but not human rPDI enhances sTF activity. The reaction mixtures were in a buffer containing 10 mM Hepes, 0.15 M NaCl, 1 mg/mL BSA, and 5 mM CaCl2 and contained sTF (10 nM), FVIIa (10 nM), and various concentrations of bovine PDI, rhPDI (0 to 100 nM) or PC/PS (80%-20% wt/wt) vesicles (0 to 10 nM). TF-FVIIa activity was measured by adding the substrate, factor X (1 μM), and measuring the amount of factor Xa generated at the end of a 10-minute reaction period in a chromogenic assay (note: y-axis is in a log scale). (B) Bovine PDI enhances prothrombin activation. Factor Xa (0.1 nM) plus FVIIIa (10 nM) were incubated for 5 minutes with a control buffer or various concentrations of bovine PDI, rhPDI, or PC/PS vesicles, and then prothrombin (1.4 μM) was added to initiate the reaction. At the end of a 2-minute reaction period, an aliquot was removed from the reaction mixture and the amount of thrombin formed was measured in a chromogenic assay. (C) Annexin V, a phospholipid binding protein, inhibits bovine PDI-mediated increased sTF activity. Bovine PDI (10 nM) or PC/PS vesicles (1 μM) was preincubated with various concentrations of annexin V for 10 minutes and then added to sTF (10 nM). TF activity was measured as described in panel A. (D) Treatment of bovine PDI with phospholipase C abolishes the enhancing effect of bovine PDI on sTF activity. Bovine PDI (10 nM) or PC/PS vesicles (1 μM) were treated with phospholipase C (0.1 U/mL) for 15 minutes before they were added to sTF. TF activity was measured as in panel A. The data shown in panels A to D represent means plus or minus SEM (n = 3-5). (E) PDI fails to bind to sTF. A CM5 sensor chip was coated with sTF and the chip was equilibrated overnight with the buffer at a flow rate of 5 μL/min. various concentrations of FVIIa or bovine PDI (10, 50, 100 nM) were passed over the sensor chip for 2 minutes (association time), followed by 10 minutes dissociation period at a flow rate of 30 μL/min. Regeneration was performed with a 3-minute pulse of 10 mM EDTA in Hepes buffer. Similar to the data shown in Figure 1E, no binding was observed between rhPDI and sTF.
affecting the activities of relipidated or cell surface TF, exactly as observed. To test this hypothesis we have examined bovine PDI for traces of PL. The activation of prothrombin by factor Xa/Va is highly sensitive to the presence of PL. As noted with sTF, addition of bovine PDI, but not rhPDI, markedly increased prothrombin activation (Figure 1B). Addition of annexin V, a phospholipid binding protein, completely attenuated the bovine PDI-mediated increase in sTF activity (Figure 1C) and prothrombin activation (data not shown), further strengthening our hypothesis that the observed enhancing effects of bovine PDI stem from contamination of the reagent with PL. Consistent with this notion is our observation that pretreatment of bovine PDI with phospholipase C, which inhibits PL function by cleaving it at the phosphate group, abolished the enhancing effect of bovine PDI on sTF. Finally, in contrast to the data obtained with immunoprecipitation studies, we found no evidence for the direct interaction of PDI with TF in Biacore binding studies (Figure 1E). In summary, the enhancing effect of bovine PDI is not limited to its effects on sTF but also extends to activation of prothrombin by factors Xa/Va. Our present data suggest that the effect of bovine PDI on sTF (and prothrombin activation) is probably an artifact due to contamination of commercial bovine PDI with traces of PL.

Hema Kothari, Prosenjit Sen, Usha R. Pendurthi, and L. Vijaya Mohan Rao

Contribution: H.K. performed the majority of the experiments described in this letter; P.S. performed the initial TF activity assays with bovine PDI and Biacore binding studies; U.R.P. and L.V.M.R. designed the research and analyzed the data; and L.V.M.R. wrote the letter.

References

To the editor:

Aberrant Ikaros, Aiolos, and Helios expression in Hodgkin and non-Hodgkin lymphoma

Ikaros transcription factors are regulators of lymphocyte development, and changes in their expression result in lymphoma development in mice.1 Although expression of Ikaros family members has been studied in different leukemias,2-4 it has not been previously reported in lymphoma. Thus, we tackle the question whether the expression of our genes of interest with the publicly available data from several research groups, we couldn’t specifically distinguish Ikaros, Aiolos, and Helios transcription factors.11 Therefore, a single gene approach of classical molecular biology is still an important complement to the microarray gene expression profiling for understanding disease processes. Taken together, our results show

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Correspondence: L. Vijaya Mohan Rao or Usha Pendurthi, Center for Biomedical Research, University of Texas Health Science Center at Tyler, 11303 US Highway 271, Tyler, TX 75708; e-mail: vijay.rao@uthct.edu or usha.pendurthi@uthct.edu.
for the first time Aiolos overexpression in primary lymphoma tissues, which could be exploited in future clinical trials.

M. Antica, L. Cicin-Sain, S. Kapitanovic, M. Matulic, S. Dzebro, and M. Dominis

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Contribution: M.A. designed and performed research and wrote the manuscript; L.C.S., S.K., and M.M., performed research and statistical analyses and contributed to the writing of the manuscript; S.D. and M.D. provided primary lymphoma samples fully characterized at the cellular and molecular level and contributed to experimental design and writing of the manuscript.

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Correspondence: Mariastefania Antica, Laboratory of Cellular and Molecular Immunology, Division of Molecular Biology, Rudjer Boskovic Institute, Bijenicka 54, 10 000 Zagreb, Croatia; e-mail: antica@irb.hr.

References


To the editor:

Eliminating the complete response penalty from myeloma response assessment

At the 2007 American Society of Hematology meeting, there were several presentations on the significant activity of combinations of novel agents with melphalan and prednisone for elderly patients with newly diagnosed myeloma. However, evaluation of the relative benefit for these different regimens is difficult given the different methods each trial used to assess progression free and event free survival. This is further complicated by the fact that several of these trials have defined progression, specifically progression from complete remission, in different ways. The frequency of complete response (CR) has dramatically increased
over the past years, reflecting significant improvements and efficacy in our treatment options. Previously, CR required no visible band in the serum protein electrophoretic pattern, while more recently, the uniform response criteria requires not only negative immunofixation, but also a normalized free light chain ratio. These more stringent definitions are important as they define disease states with lower tumor mass, and in most studies are associated with improved duration of remission and overall survival. However, many nonlaboratory-based practitioners may not realize that immunofixation is a qualitative test and is subject to interpretation. It requires manual inspection of the immunofixation pattern to determine whether a discrete monoclonal band is visualized. This can cause variation in reported results over successive months between “absent,” “possible,” or “small monoclonal protein.” Moreover, high-dose chemotherapy or other treatment modalities that induce complete remissions are often associated with the development of posttransplantation monoclonal or oligoclonal bands that do not appear to be the same as the original M-protein, and yet can be difficult to distinguish from either relapse or normal hematopoietic recovery.

These issues regarding the accuracy of immunofixation do not impact decisions regarding therapy, as few clinicians reinitiate therapy in an asymptomatic patient with only biochemical evidence of progression. It does however cause difficulty when large groups report outcomes of trials in which there are high rates of CR. Progression from a CR requires only conversion from immunofixation negative to immunofixation positive in a single instance, while progression from any other disease response state requires an increase in the M-protein by 25% and an M-protein increase greater than 0.5 g/dL. Because the duration of response in a patient who achieves a CR is measured from the time a complete response is verified to the time the monoclonal protein reappears, it is possible to have shorter response durations among CR patients than in those patients who achieve only a partial response.

This difference in definition of progression between CR and other responses results in a distortion of the therapeutic benefit of a complete response and results in artificial reporting that reduces the value of the information for practicing clinicians. One potential way to circumvent this problem is to report response rates from these trials as an amalgamation of CR plus very good partial response (VGPR, > 90% reduction in the M-protein). Progression in the setting of a CR would then be defined as the presence of a protein level greater than a VGPR, which would by definition require that the M protein be greater than 10% of its original value. This would eliminate labeling a patient as having progressive disease based on reappearance of the band by immunofixation and would align it to the recently introduced and accepted VGPR. We would, therefore, propose that cooperative groups consider reporting CR plus VGPR as an aggregate percentage, and that the time to progression be defined as the time from the date of response to the date at which the M protein exceeds VGPR values, that is, greater than 10% of the original M protein size. Use of this approach will provide a clinically useful gauge for the durability of remission for patients who achieve a major response to therapy, and will eliminate the paradoxically shorter response duration for patients who achieve complete remissions.

Sagar Lonial and Morie A. Gertz

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Correspondence: Dr Sagar Lonial, Emory University School of Medicine, Building C, Room 4004, 1365 Clifton Rd, Atlanta, GA 30322; e-mail: sloni01@emory.edu.
Bovine protein disulfide isomerase-enhanced tissue factor coagulant function: is phospholipid contaminant in it the real culprit?

Hema Kothari, Prosenjit Sen, Usha R. Pendurthi and L. Vijaya Mohan Rao