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Fibrin β^{15-42} domain is cryptic in intact fibrinogen: comment on the study by A. Sahni *et al.*

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Dear Editor,

With interest we read the article by Abha Sahni and coworkers; "The VE-cadherin binding domain of fibrinogen induces endothelial barrier permeability and enhances transendothelial migration of malignant breast epithelial cells" in the edition of August 1st, 2009.[1] Sahni *et al.* showed that addition of intact fibrinogen induces an increase in endothelial permeability. This increase was shown to be mediated via binding of fibrinogen domain β^{15-42} to VE-cadherin on endothelial cells. Transendothelial migration of malignant breast cancer cells was increased by fibrinogen. The data provide novel insight in transendothelial migration of malignant cells, which require fibrinogen domain β^{15-42} and VE-cadherin binding.

Sahni *et al.* showed a significantly increased permeability of human endothelial cells using 300 nM (0.1 g/L) fibrinogen. This is a surprising finding, since this is below the fibrinogen concentrations in the human circulation. In our own experiments, 50 and 200 nM intact fibrinogen showed no increase in permeability of the endothelial monolayer, using highly purified intact fibrinogen fractions (unpublished). Published data on higher fibrinogen concentrations do not show a consensus on whether the intact protein increases vascular permeability. Tyagi *et al.* showed that at pathologically high levels, fibrinogen ($\geq$12 μM) can increase permeability of the endothelial monolayer, whereas physiological concentrations (6–12 μM) had no effect.[2] Here, Sahni *et al.* showed an induction of endothelial permeability using 300 nM fibrinogen. Referring to the human circulation, it would be surprising if fibrinogen concentrations beneath physiological levels would be able to increase permeability. Therefore, we carefully compared the experiments and proposed mechanism.

The fibrinogen-induced permeability was investigated using peptides and blocking antibodies. Sahni *et al.* showed an impaired fibrinogen-induced permeability after addition of antibody T2G1 excess. This suggests that the antibody T2G1 bound intact fibrinogen and thereby impaired endothelial permeability. However, Kudryk *et al.* showed that the monoclonal antibody T2G1 only binds β^{15-21} on fibrin and its degradation products, whereas the binding to intact fibrinogen was shown to be ~250 times lower.[3] Moreover, peptide β^{15-42} was shown to mimic fibrinogen-induced permeability, but the control peptide had no effect.[1] As stated in the introduction, domain β^{15-42} is cryptic in fibrinogen and exposed after cleavage of fibrinopeptide B or proteolytic degradation.[1,4] Domain β^{15-42} is also being exposed after thrombin-independent fibrinogen matrix assembly.[5] In the methods of the article, it was described that intact soluble fibrinogen was added to the apical side of the endothelial monolayer. The question rises whether fibrinogen domain β^{15-42} was exposed and bound to endothelial VE-cadherin to increase permeability. The role of VE-cadherin regulating permeability in the endothelial cell junctions has been extensively studied.[6] Moreover, as shown by Ge *et al.*, relatively low concentrations fibrin degradation products increase endothelial permeability. It was shown that 0.5–2.0 μM (0.05–0.2 g/L) fragment D caused a doubling of the transendothelial ^{125}I-albumin clearance in 2 hr.[7] Healthy individuals have D-dimer concentrations with a maximum of 0.0005 g/L. Taken together, these findings strongly suggest the presence of fibrin monomers or fibrin degradation products in the fibrinogen sample used by Sahni *et al.* Alternatively, the exposure of cryptic domains within the fibrinogen molecule by conformational changes may contribute to the exposure of fibrin specific epitopes, such as is also observed after fibrinogen binding to plastic culture plates.

The mechanism proposed by Sahni *et al.* is that tumor-associated fibrinogen can facilitate transendothelial migration of malignant cells. Intact fibrinogen would increase vascular permeability via binding of fibrinogen domain β^{15-42} to endothelial VE-cadherin. However, domain β^{15-42} was shown to be cryptic in intact fibrinogen.[3] In our opinion, not intact fibrinogen but fibrin or its degradation products are responsible for the observed effects. This sheds a new light on the data. Palumbo *et al.* showed that fibrin(ogen) is an important determinant of the metastatic potential of tumor cells. In wild-type mice, a 20-fold reduction of tumor metastasis was seen after addition of the thrombin-inhibitor hirudin.[8] In addition to a contribution of fibrinogen to platelet-microthrombi, which may provide protection to tumor cells against innate immune surveillance systems,[9] Sahni *et al.* proposed that tumor-associated fibrinogen can facilitate transendothelial migration of malignant cells. Our addition to the latter proposed mechanism would be that tumor-associated fibrinogen is converted to fibrin monomers or fibrin degradation products. Thereafter, it acts as a bridging molecule between tumor cells and endothelial cells, after which transendothelial migration can take place. The bridging of fibrin to VE-cadherin on endothelial cells is mediated via domain β^{15-42} on the molecule.

In summary, findings demonstrating that relatively low concentrations of intact fibrinogen increase permeability

should be reconsidered. Domain β^{15-42} is exposed after thrombin cleavage and degradation of fibrin, thereafter the fibrin specific antibody T2G1 can bind. The results shown by Sahni *et al.* strongly suggest the presence of fibrin specific epitopes, which bear impact on the interpretation of the data. In this view, not intact fibrinogen but fibrin monomers or fibrin degradation products form the bridging molecule between tumor and endothelial cells. Not withstanding, the results showing malignant cell migration mediated by domain β^{15-42} fibrin are novel and add to the existing models on extravasation of metastatic tumor cells. It would be wonderful when such new insight would result in improvement of therapeutic interventions.

Yours sincerely,
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Author's reply to Weijers *et al.* "Fibrin β^{15-42} domain is cryptic in intact fibrinogen: comment on the study by A. Sahni *et al.*"

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Dear Editor,

We thank Drs. Weijers, Koolwijk, van Hinsbergh and van Nieuw Amerongen for their interest in our recent publication, "The VE-cadherin binding domain of fibrinogen induces endothelial barrier permeability and enhances transendothelial migration of malignant breast epithelial cells" that appeared in the August 1st issue of the *International Journal of Cancer* in 2009.[1] Moreover, we appreciate the concerns raised by Weijers *et al.*[2] that the effects we observed on endothelial permeability and malignant breast epithelial cell transendothelial cell migration were due to thrombin/proteolytic conversion of the exogenously added fibrinogen to fibrin monomer and/or fibrin(ogen) degradation products and not due to the exposure of the cryptic vascular endothelial (VE)-cadherin binding domain located at residues 15–42 on the β chain of intact fibrinogen. Although a role for VE-cadherin in regulating endothelial cell permeability is well established,[3] a role for intact fibrinogen in the absence of its conversion to fibrin and/or fibrin(ogen) degradation products to promote endothelial cell permeability would be unexpected.

When we initiated these studies, we also expected that only fibrin monomer and fibrin(ogen) degradation products, as previously reported in the literature, and as cited by Weijers *etal*,[2] would induce permeability of barrier endothelium. The commercially purchased highly purified fibrinogen used in all of our studies is subjected to additional rigorous quality control as described.[1] Although small amounts of fibrin I and fibrin II as

well as some fibrin(ogen) degradation products are found in commercial sources of purified human fibrinogen prepared from pooled plasma, we are convinced there is negligible contamination with fibrin monomer and/or fibrin(ogen) degradation products to account for the effects observed.[1]

Weijers *et al.*[2] raise the possibility that intact fibrinogen is converted to fibrin by thrombin when added to the conditioned medium of the cultured endothelial cells. To address this concern, we show additional data collected from control experiments over the course of our studies (Fig. 1). To determine whether thrombin activity affected permeability induced by intact fibrinogen, we assessed FITC-Dextran flux across the endothelial barrier as described[1] in the absence and presence of the specific thrombin inhibitor, hirudin. As shown in Figure 1*a*, 1 Unit/ml hirudin had no effect on the flux of FITC-Dextran across the endothelial barrier ($p = 0.14$, Student's *t*-test), indicating that active thrombin was not participating in fibrinogen-induced endothelial permeability. Higher concentrations of hirudin (up to 5 Units/ml) also had no effect on endothelial barrier permeability (unpublished data).

To address the suggestion that the soluble, intact fibrinogen was converted to fibrin and/or fibrin(ogen) degradation products by the malignant breast epithelial cells, we show data collected by Dr. Brian Rybarczyk that was published in his doctoral dissertation.[5] Iodinated fibrinogen exposed to MDA-MB-231 cells for 18 hr remained intact at 340 kDa; it was not cleaved by thrombin or crosslinked to form fibrin(ogen) polymers, nor were typical proteolytic degradation products (e.g., plasmin-derived fragments D and E) observed (Fig. 2*a*). Data published from our laboratory indicate that fibrinogen added exogenously to fibroblasts (please see Fig. 7 in Ref. 4) or endogenously produced by A549 lung epithelial cells (please see Fig. 9 in Ref. 4) remains intact. Additional data from our laboratory suggest that cell-associated fibrinogen interacts with cell surface integrins $\alpha5\beta1$ and $\alpha v\beta3$, either directly or indirectly through association with fibronectin and with cell surface heparan sulfate proteoglycans (HSPGs).[5–7]

It is widely accepted by others[2,8–10] and us[11] that residues defined by fibrinogen-β^{15-42} are cryptic in intact fibrinogen—the fibrin-specific monoclonal antibody T2G1[8] will not bind to its epitope, defined by residues β,[15–21] when intact fibrinogen is presented either in solution or adsorbed to a solid surface. However, we have shown that when highly purified intact fibrinogen is added to the solution phase of metabolically active cells in culture, fibrinogen assembles into the extracellular matrix in the absence of proteolytic cleavage to fibrin, fibrin(ogen) degradation products, or processed into higher ordered structures such as covalently cross-linked fibrin(ogen).[4,6,7,12] This was observed using both primary cells (human foreskin fibroblasts and human umbilical vein endothelial cells) as well as human cell lines, including lung type II-like A549 epithelial cells, and nonmalignant (HBL100) and malignant (MDA-MB-231) breast epithelial cells. Moreover, we have shown conclusively that β^{15-21} exposed in fibrillar matrix fibrinogen is recognized by monoclonal antibody T2G1 (Fig. 2, reproduced with permission from *Blood*, 2003;102:4035–43; Cover photo).[7]

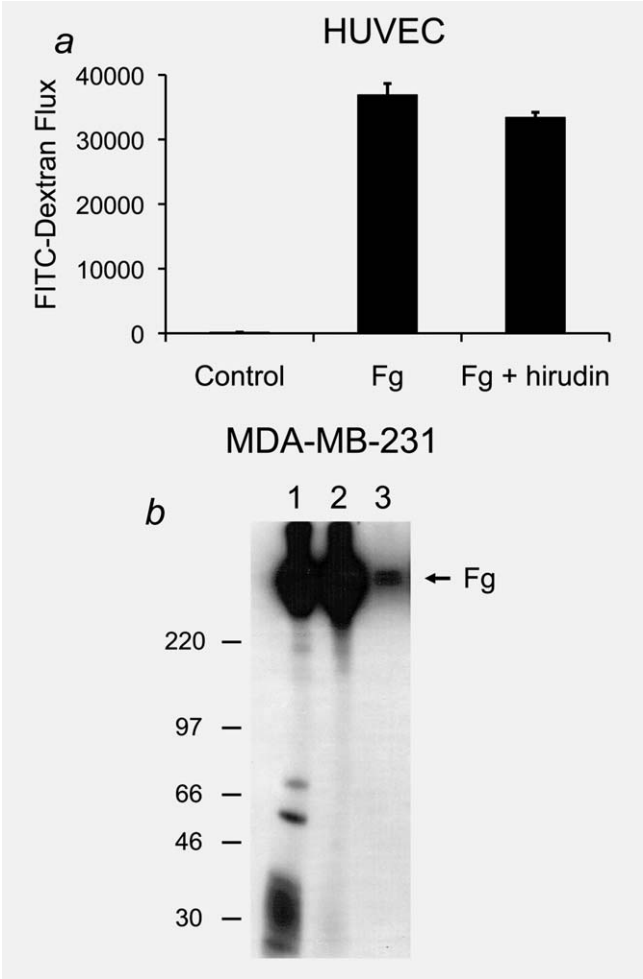

Figure 1. Neither thrombin cleavage (Panel *a*) nor protease degradation (Panel *b*) of intact fibrinogen occurs in the presence of endothelial (Panel *a*) or breast cancer cell (Panel *b*) conditioned media. Panel *a*, Intact fibrinogen (Fg) (30 nM), in the absence and presence of the specific thrombin inhibitor hirudin (1 Unit/ml), was added to the apical chamber of human umbilical vein endothelial cells (HUVEC) grown to confluency on Millicell™ inserts and FITC-Dextran flux across the endothelial barrier was measured at 45 min as described by Sahni *et al.*[1] Panel *b*, Iodinated fibrinogen (5 μg/ml) was added to the supernatant of confluent monolayers of the highly invasive MDA-MB-231 breast cancer cell line, and incubated for 18 hr. The fibrinogen was recovered as described[4] from the serum containing conditioned medium (Lane 2) and cell-associated (Lane 3) fractions and, along with the starting material (Lane 1), resolved under nonreducing but denaturing conditions on a 4–12% gradient polyacrylamide gel. The migration of molecular weight markers in kDa is indicated.

Palumbo *et al.*[13–17] have performed elegant experiments in mutant mice to show that plasma fibrin(ogen), thrombin and platelets are all important in promoting metastasis of certain

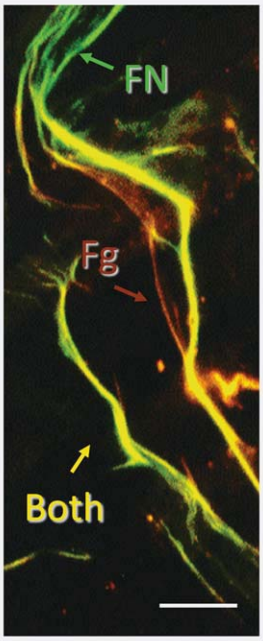

Figure 2. The cryptic fibrin-like β^{15-21} epitope is exposed in matrix fibrinogen fibrils that colocalize with fibronectin fibrils. Confluent monolayers of human foreskin fibroblasts were treated with purified plasma fibrinogen at 40 μg/ml for 24 hr. Fibrinogen (Fg) matrix fibrils were detected with monoclonal antibody T2G1 (red fluorescence), which recognizes the neo-NH_2-terminus (β^{15-21}) of the fibrin β chain, and fibronectin (FN) matrix fibrils (green fluorescence) were detected with polyclonal anti-human fibronectin IgG. Very few isolated fibrils of fibronectin (green staining) or fibrinogen (red staining) are found in the matrix; the majority of fibrinogen colocalizes (Both) with fibronectin fibrils (yellow staining), indicating that the cryptic β^{15-21} epitope is exposed in fibrinogen matrix fibrils distributed along the length of fibronectin matrix fibrils. The scale bar represents 25 μm. (Reproduced with permission from Rybarczyk, *et al.,* Matrix-fibrinogen enhances wound closure by increasing both cell proliferation and migration, 2003, 102, 4035–43, *Blood*). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

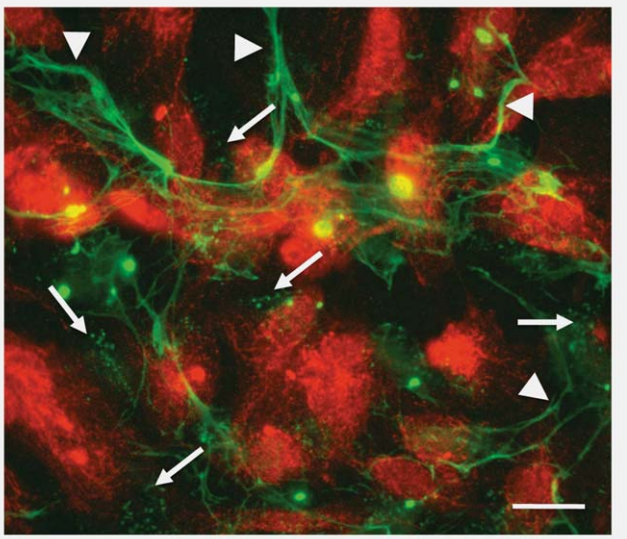

Figure 3. Fibrinogen binds to cell surface receptors and assembles into the subendothelial fibrillar matrix of endothelial cells. Human umbilical vein endothelial cells were grown to confluence on gelatin-coated glass coverslips then treated for 24 hr with 30 nM purified plasma fibrinogen conjugated to Oregon Green (green fluorescence). The cells were fixed, permeabilized and stained with antibodies to fibroblast growth factor-2 (red fluorescence). After immunostaining, the coverslip was mounted "upside down" on a microscope slide such that the basolateral aspect and the subendothelial extracellular matrix appear on "top" of the cells. Epifluorescence microscopy revealed that fibrinogen binds to endothelial cell-cell junctions in a punctate pattern (green fluorescence), consistent with fibrinogen binding to its intercellular surface receptor, VE-cadherin (arrows). Fibrinogen also assembles to a limited extent into the subendothelial fibrillar extracellular matrix as denoted by the strands of green fluorescence (arrowheads). The scale bar represents 25 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cancers to the lung. Pertinent to this response are their results showing that, in fibrinogen-deficient mice, the number of metastatic foci of B16-BL6 melanoma and Lewis Lung carcinoma was greatly reduced in lung.[13,14] Weijers *et al.*[2] raise the point that when Palumbo and colleagues performed the above experiments in the absence or presence of the specific thrombin inhibitor hirudin, lung metastasis was reduced 20-fold in hirudin-treated wild-type mice (please see Fig. 5 in Ref. 13). However, hirudin also decreased lung metastasis comparably in fibrinogen-deficient mice (please see Fig. 5 in Ref. 13), suggesting that the prometastatic affects of thrombin were, for the most part, fibrinogen independent. It is well known that thrombin contributes to the severity of cancer progression by promoting tumor angiogenesis, cancer cell proliferation and metastasis by mechanisms other than just thrombin generation of fibrin.[18]

The neo-NH_2-terminus of the fibrin β chain, defined by residues 15–42, participates in clot formation and also has been implicated in a number of important cellular responses including cell spreading,[19] stimulating cell proliferation,[20] binding to VE-cadherin to support angiogenesis,[21] binding to endothelial cell HSPGs[22] and inducing cell migration during wound repair.[7] We have shown that a cryptic heparan binding domain maps to β^{15-42} residues of fibrin(ogen).[23] Because the VE-cadherin binding domain and heparan binding domain on fibrin(ogen) overlap on the fibrinogen β chain, we cannot rule out the possibility that endothelial cell surface HSPGs play a role in intact fibrinogen-induced endothelial permeability. However, we consider this the least favorable interpretation of our data because heparin-dependent binding

of fibrin monomer to endothelial cells occurred only when the endothelial cells were fixed to the ELISA plate during the cell binding assay. In contrast, fibrinogen bound poorly to fixed endothelial cells in this cell binding assay.[22] Furthermore, we have shown that fibrinogen assembly into the extracellular matrix requires binding to the cell surface before deposition into the matrix (please see Fig. 9 in Ref. 5). Such cell surface binding is integrin dependent, requiring fibrinogen Aα-chain RGD sequences. However, this cell surface binding is not HSPG dependent in that desBβ^{1-42}-fibrinogen, also known as fibrinogen-325, still assembles into matrix fibrils (please see Figs. 3–5 in Ref. 7). Moreover, only metabolically active cells are capable of assembling fibrinogen into mature matrix fibrils (please see Fig. 1 in Ref. 24). Taken together, these published data support our conclusion that in the absence of malignant breast cancer cells, endothelial barrier permeability occurs only after intact fibrinogen binds to integrins on the endothelial cell surface. Binding induces a conformational change exposing the fibrinogen β^{15-42} domain, thus allowing it to bind to VE-cadherin. This conclusion is further supported by the photomicrograph (Fig. 3 and cover photo for Sahni *et al.*[1]) showing that fibrinogen assembles into subendothelial matrix fibrils and binds to the surface of endothelial cells at cell-to-cell junctions where VE-cadherin is found.

We agree that fibrin and thrombin play significant physiologic roles in cancer metastasis. However, the exact structure of fibrin(ogen) and fibrin(ogen) degradation products that promote endothelial barrier permeability is poorly understood. We pose the intriguing possibility that subphysiological levels of fibrinogen modulate cell responses in the microenvironment where fibrinogen, tumor cells and endothelium interact. Extensive research from our laboratory has contributed to elucidating molecular mechanisms regulating gene expression, protein biosynthesis and assembly, and function of fibrinogen produced by extrahepatic epithelium from the lung, prostate and breast using adenocarcinoma cell lines.[4,5,25–30] Others have reported expression of the fibrinogen genes and secretion of fibrinogen protein by intestinal and cervical epithelial cancer cell lines.[31,32] Extrahepatic expression of fibrinogen occurs in vivo, as demonstrated in animal models of *Pneumocystis carinii* pneumonia, and by gene expression profiling of biopsy samples from patients with lung or breast cancer.[33–39] The functional significance of fibrinogen expression by extrahepatic epithelium is likely influenced more by the cellular context in which the fibrinogen is found rather than by its overall plasma concentration.

In summary, we stand by our interpretation that exposure of the VE-cadherin binding domain in intact fibrinogen induces endothelial cell permeability and promotes transendothelial migration of malignant breast cancer cells *in vitro*.[1] Furthermore, it is reasonable to hypothesize that small amounts of fibrinogen produced endogenously by breast cancer cells contribute to tumor cell-endothelial cell interactions to promote breast cancer metastasis *in vivo*. The striking observation that intact fibrinogen was unable to induce barrier endothelial permeability in the presence of MCF-10A nonmalignant breast epithelial cells (please see Fig. 6 and Discussion in Ref. 1) led us to further hypothesize that nonmalignant breast epithelial cells bind fibrinogen β^{15-42} sequences, likely through cell surface HSPGs, rendering the VE-cadherin binding domain at fibrinogen β^{15-42} inaccessible. The ability of nonmalignant breast epithelial cells to bind fibrinogen β^{15-42} and the loss of such binding capacity by malignant breast cancer cells suggests a molecular mechanism by which premalignant breast epithelial cells transform to a highly aggressive metastatic breast cancer phenotype. Further studies in animal models of breast cancer metastasis will permit us to test the validity of these hypotheses.

Yours sincerely,
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