Role of von Willebrand factor in tumor metastasis

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Abstract

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that plays a critical role in primary hemostasis, allowing the adhesion of platelets to the exposed subendothelium. The key role played by VWF in platelet adhesion suggests a potential implication in various pathologies where this process is involved. In cancer metastasis development, tumor cells interact with platelets and the vessel wall to extravasate from the circulation. A number of potential receptors for VWF have been identified on tumor cells such as glycoprotein Ib or the αIIbβ3 and αvβ3 integrins and direct interactions between VWF and tumor cells have been reported. To address the role of VWF in an experimental metastasis model, we compared the formation of pulmonary metastatic foci in C57BL/6J wild-type and VWF-null mice following I.V. injection of murine melanoma B16-BL6 cells or Lewis lung carcinoma cells. Surprisingly we found a significant increase in the number of pulmonary metastatic foci in VWF-null mice. Restoration of VWF plasma levels by co-injection of VWF with the tumor cells led to the correction of this pro-metastatic phenotype. In vitro analysis revealed that VWF did not influence tumor cell proliferation or invasion but induced cellular death. This result was confirmed in vivo where analysis of the early survival of tumor cells in the lungs revealed that the presence of VWF led to a decreased survival of these cells during the first 24 hours after injection. Our results suggest that VWF plays a role in tumor metastasis, independently of its role in hemostasis.

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1. Abbreviations

VWF: von Willebrand factor; GP: glycoprotein; LLC: Lewis Lung Carcinoma.

2. Introduction

Von Willebrand factor (VWF) is a multimeric plasma glycoprotein that plays a prominent role in primary hemostasis [1]. In order to perform this function, VWF binds to receptors on platelets, the glycoproteins (GP) Ib and IIb/IIIa, and on exposed subendothelium forming a bridge at the site of vascular injury, leading to the formation of the platelet thrombus [2]. The molecular mass of VWF can range from 500,000 for the dimer to over $10^6$ daltons for the large multimers. VWF is synthesized by endothelial cells and megakaryocytes [3]. Megakaryocyte-derived VWF is stored in the α-granules of platelets whereas endothelial cell-derived VWF can be either released constitutively in the plasma or stored in specialized organelles called the Weibel-Palade bodies. Upon activation of the endothelial cells by various agonists, the Weibel-Palade bodies will release mostly high molecular weight multimers of VWF, the forms that are the most biologically active [4]. The role of VWF is particularly important at high shear rates, conditions encountered in arterioles and in the microcirculation. In the coagulation process, VWF acts as a carrier for coagulation factor VIII (FVIII) and protects it against inactivation by proteases. Any qualitative or quantitative defect of VWF leads to von Willebrand disease, the most common bleeding diathesis. Von Willebrand disease can be classified in three major subtypes reviewed by Ewenstein. Types 1 and 3 are caused by mild and severe quantitative defects in VWF, respectively, whereas type 2 is due to qualitative abnormalities [5].

Because of its important involvement in platelet adhesion and thrombus formation, VWF is likely to play a role in diseases in which these two physiological functions play a role, i.e. atherosclerosis, thrombotic thrombocytopenic purpura, disseminated intravascular coagulation, stroke, cancer metastasis, sickle cell disease and glomerular nephritis. Tumor metastasis...
formation is a multi-sequential dynamic process in which tumor cells first establish interactions with the platelets and the vessel wall before colonizing secondary sites. There is evidence that successful metastasis depends on the ability of tumor cells to interact with platelets [6]. Platelets have been proposed to protect tumor cells from premature clearance by the immune system [7]. Alternatively, the thrombus resulting from platelet-tumor cell interactions could bind more easily to the vessel wall and therefore be more invasive [7–9]. VWF represents a potential candidate to mediate platelet-tumor cell interactions. Its multimeric nature enables it to bind several ligands simultaneously and a number of reports have described a direct interaction between VWF and tumor cells [10–13]. These interactions involve receptors such as GPIb [14], or the integrins αIIbβ3 [10] and αvß3 [13,15].

Another link between VWF and cancer comes from the observation of increased VWF antigen levels in patients with ovarian, bladder and colon cancers. These increased levels usually correlate with poor prognosis and metastatic disease [16–18]. Furthermore, VWF has been shown to increase the adhesion of tumor cells to endothelial cells in a co-culture system [19]. Finally, antibody-mediated VWF inhibition in mice resulted in a decreased metastasis development induced by various tumor cell lines [20].

In view of these different reports, we decided to investigate the direct role of VWF in tumor metastasis using VWF-deficient mice. Following the in vivo assessment of the metastatic response using two different murine tumor cell lines, we investigated the mechanism underlying VWF involvement in tumor cells metastatic phenotype.

3. Material and methods

3.1. Mice

The VWF-deficient mice [21] and wild-type mice used in this study were on a C57BL/6 background and were used between 6 and 10 weeks of age. Housing and experiments were done as recommended by French regulations and experimental guidelines of the European Community.

3.2. Proteins

Purified plasma-derived human VWF was a kind gift from Dr C. Mazurier, LFB, Lille, France and was stored in small aliquots at –80°C.

3.3. Cell culture

The two murine cell lines, the melanoma B16-BL6 and the Lewis Lung Carcinoma (LLC) were obtained from Dr. M. Gebbink (University Medical Center Utrecht, The Netherlands). They were cultured in Dulbecco’s modified Eagle medium (DMEM) (Sigma, Saint Quentin Fallavier, France), supplemented with 10% fetal calf serum from Biowest (Abcys, Nuaille, France), penicillin, streptomycin and glucose from Gibco (Invitrogen, Cergy-Pontoise, France), in 5% CO₂.

3.4. Experimental metastasis model

Subconfluent tumor cells were washed with PBS, detached by 0.5 mM EDTA, washed in serum-containing medium and then resuspended in cold serum-free medium. The cells were kept on ice until transplanted in mice. B16-BL6 cells (5 × 10⁴ cells in 200 µl) or LLC cells (1.5 × 10⁵ cells in 200 µl) were injected into the lateral tail vein of the mice. After 14 days, the mice were euthanized; the lungs were removed and rinsed in 0.9% sodium chloride. The lungs were separated into individual lobes and the number of metastatic colonies on the surface was counted by an investigator unaware of the mouse genotype. In one set of experiments, the effect of plasma VWF was tested by co-injecting human VWF (10 µg/ml, final concentration) together with B16-BL6 cells in VWF-deficient mice.

3.5. Labeling of B16-BL6 cells with [125I]-iododeoxyuridine and quantitative analysis of their distribution after injection in the circulation

Melanoma B16-BL6 cells (10⁶ cells) were plated on 100 mm dishes, and grown for 24 h in DMEM containing 10% serum. Then, 1 µCi/ml 5-[125I]-iododeoxyuridine (ICN Pharmaceuticals France SA, Orsay, France) was added to the medium and the cells were incubated for an additional 24 h [22]. After washing with PBS, the cells were detached as described above. [125I]-iododeoxyuridine-labeled B16-BL6 cells were then resuspended in cold serum-free medium, and 2 × 10⁵ cells were injected into the lateral tail vein of mice. The fate of these labeled tumor cells in the first 24 h following injection was determined as described [13]. Since the label from dead cells is rapidly excreted from the body, the residual radioactivity corresponds to live cells exclusively.

3.6. Apoptosis assays

To induce cell apoptosis, cells grown to 80% confluency in 35-mm dishes or in Lab-Tek II 8-well slides were placed in serum-free medium in the presence of VWF (10 µg/ml) or vehicle for the indicated time periods. Apoptotic cells were analyzed by cell death and annexin V binding. For the cell death assay, floating and adherent cells in the 35-mm dishes were
separately resuspended in 0.4% trypan blue and cells that had taken up this dye were considered non viable. The percentage of dead cells was calculated as a ratio of floating dead cells to the total number of cells/culture dish. For the annexin V binding assay, the annexin V-FITC fluorescence microscopy kit from Becton Dickinson was used according to manufacturer’s protocol (Becton Dickinson, Le-Pont-de-Clai, France).

3.7. Proliferation assay

To measure cell proliferation, 5000 B16-BL6 cells were seeded in a 96-well microtiter plate in 100 μl serum-containing medium and in the presence of VWF (10 μg/ml) or vehicle. After 24, 48 or 72 h of incubation at 37°C, cell proliferation was assessed using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit from Promega (Promega, Charbonnières, France). Briefly, 20 μl of a tetrazolium compound was added to each well and incubated for 1 h. Optical density was then read at 490 nm in Power Wave 340 × microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). Wells containing medium only were used as blanks.

3.8. Matrigel invasion assay

B16-BL6 cell invasiveness was assessed in vitro using modified Boyden chambers. Inserts containing an 8 μM pore size polycarbonate membrane covered with a thin layer of ECMatrix™ were placed in a 24-well tissue culture plate (Cell Invasion assay kit, Chemicon International Ltd, Hampshire, United Kingdom). Cells (1 × 10^5) were added to the top of the chamber in serum-free medium in the presence of VWF (10 μg/ml) or vehicle. Serum-containing medium was used as chemoattractant in the lower chamber. After 24 h, cells that had migrated to the underside of the filters were stained and dissolved in 10% acetic acid. Optical density was read at 560 nm.

3.9. Cell migration assay

B16-BL6 chemotaxis cell migration was measured in vitro using the QCM™ 24-well colorimetric cell migration assay from Chemicon. 24 h before the assay, cells were starved by removing the serum from the medium. After harvesting the next day, cells were resuspended in serum-free medium and 0.7 × 10^5 cells were added to the top of the chamber. In this assay, the 8 μM pore size polycarbonate membrane is not coated with any basement membrane. Serum-containing medium was used as chemoattractant in the lower chamber. After 24 h cells that had migrated to the underside of the filters were stained. The stain was extracted and optical density was read at 560 nm. Wells without cells were taken as controls.

3.10. Data analysis and statistics

Data are presented as mean ± SEM or mean ± SD as indicated in the figure legends. Statistical analyses were performed using either the Student’s unpaired t-test or the non-parametric Mann Whitney U-test, as indicated in the figure legends, with the Statview program (Statview version 5, SAS Institute Inc, Cary, NC, USA).

4. Results

4.1. Role of VWF in experimental metastasis

To test the hypothesis that VWF is a determinant of the metastatic potential of tumor cells, we used an experimental pulmonary metastasis model using two highly metastatic murine cell lines, the melanoma B16-BL6 and the LLC cells. Injection of B16-BL6 tumor cells in the tail vein of mice resulted in the development of dark and easily visible metastatic colonies on the lungs. Similar observations were done after injection of LLC cells with the difference that the metastatic colonies appeared white (Figure 1b). The development of pulmonary metastatic foci both in wild-type and VWF-deficient mice shows that VWF is not absolutely required for haematogenous metastasis. However quite unexpectedly, we observed a significantly increased number of metastatic foci in VWF-deficient mice as compared to their wild-type littermates (Figure 1a). This observation was confirmed in independent experiments and for both cells types. Two independent experiments performed with B16-BL6 cells are represented in Figure 1a with each point representing the number of metastatic colonies counted on the lungs of one mouse. In experiment 1, an average of 67.7 ± 9.5 metastatic foci was counted in VWF +/+ mice (range 17–129), and 111.00 ± 6.6 in VWF −/− mice (range 77–144) (p = 0.046). In experiment 2, we counted an average of 34.5 ± 8 metastatic foci in VWF +/+ mice (range 5–86), and 57.7 ± 4.3 in VWF −/− mice (range 34–84) (p = 0.041). For LLC cells, we counted an average of 4.1 ± 0.6 foci in VWF +/+ mice (range 1–9), and 20.5 ± 4.5 in VWF −/− mice (range 2–61) (p = 0.0006). Histologically, there was no difference between the B16-BL6 pulmonary foci obtained in either genetic background (not shown).

To check whether this rather surprising result was the direct consequence of VWF deficiency, we next co-injected human recombinant VWF (10 μg) together with B16-BL6 cells in VWF-deficient mice. This restoration of VWF plasma levels resulted in
Fig. 1. Effect of VWF deficiency on experimental pulmonary metastasis formation: B16-BL6 melanoma cells (5 × 10⁴) or LLC cells (1.5 × 10⁵) were injected intravenously in wild-type (VWF+/+) and VWF-deficient (VWF−/−) mice by tail-vein injection. Lungs were isolated after 14 days. (a) Number of pulmonary nodules in VWF+/+ and VWF−/− mice in two independent experiments using B16-BL6 cells: Experiment 1: n = 15 for VWF+/+ and n = 13 for VWF−/−, p = 0.0046; Experiment 2: n = 16 for VWF+/+ and n = 14 for VWF−/−, p = 0.041. For the LLC experiment, n = 15 for VWF+/+ and n = 13 for VWF−/−, p = 0.0006 (Mann Whitney, U2-tailed). With both cell types, the number of pulmonary nodules was significantly higher in VWF-deficient mice. (b) Representative examples of lungs with metastatic pulmonary foci of B16-BL6 melanoma cells and LLC cells in VWF+/+ and VWF−/− mice 14 days after cell injection. Figure from Terraube et al. [13], with permission. © 2006 International Society of Thrombosis and Haemostasis.

Fig. 2. Effect of VWF on B16-BL6 cell proliferation. B16-BL6 cells were incubated for 24, 48 or 72 h in the presence of 10 μg/ml of VWF (squares) or vehicle (circles). Proliferation was measured at these different time-points by the addition of tetrazolium compound and by reading the absorbance at 490 nm. Results are shown as mean±SD of triplicates. Absorbance of medium alone has been subtracted.

4.2. Effect of VWF on B16-BL6 behavior in vitro

Since VWF seems to exert a direct effect on experimental metastasis, we addressed the question of whether it could affect tumor cell behavior in a series of in vitro tests. In a first attempt to answer this question, we measured B16-BL6 cells proliferation in the presence or absence of VWF. As shown in Figure 2, VWF does not influence proliferation of B16-BL6 cells. Similarly we could not detect any effect of VWF on cell migration (Figure 3a) or invasiveness through a basement membrane (Figure 3b).

We next checked the capacity of VWF to affect serum-deprivation-induced apoptosis. Incubation of B16-BL6 cells in serum-free medium for 8, 24 and 48 h caused a gradual increase in the number of floating dead cells, an effect that was significantly exacerbated by the presence of VWF in the medium after 24 or 48 h (Figure 4). After 24 h there was a
Fig. 4. Effect of VWF on cellular death. B16-BL6 cells grown to 80% confluency were serum starved and incubated with 10 μg/ml of VWF or vehicle for 24, 48 or 72 h. The percentage of dead cells at these different time-points was evaluated by trypan blue exclusion assay. Results are expressed as mean±SEM of triplicates. *p < 0.05.

Fig. 5. Effect of VWF on annexin-V binding. B16-BL6 cells grown to 80% confluency were serum starved and incubated with 10 μg/ml of VWF (c and d) or vehicle (a and b) for 48 h. Adherent cells were stained with Annexin V-FITC and observed under phase contrast (a and c) or fluorescence microscopy (b and d). Annexin V binding appears stronger in VWF-treated cells.

4.3. Early fate of tumour cells in vivo

Considering that VWF seems to affect tumor cell survival in vitro, we next assessed the early fate of B16-BL6 in vivo during the first 24 h following injection in the mice. Radiolabelled tumor cells were injected intravenously in wild-type and VWF-deficient mice. 15 minutes after injection, the vast majority of melanoma cells had disappeared from the circulation since only about 1% of B16-BL6 cells remained in blood of wild-type and VWF-deficient mice (data not shown). At the same time, 70 to 85% of melanoma cells were already detected in the lungs, independently of the mouse genotype (Figure 6), while only 3.5% and 0.3% of the radioactivity was found in the liver and spleen respectively. With time, the number of viable cells in blood and organs decreased regularly. In the lungs, after 4 h the number of cells in VWF-deficient mice appeared larger than in wild-type mice, but this difference was not statistically significant (VWF−/−: 52.3% ± 3.4, VWF+/+: 43.0% ± 4.4, p = 0.13). However after 24 h, it appeared clearly that more B16-BL6 cells were present in the lungs of VWF-deficient mice compared to wild-type mice (VWF−/−: 9.96% ± 0.59, VWF+/+: 6.10% ± 0.92, p = 0.0078), confirming the in vitro data suggesting that the absence of VWF favours survival of tumour cells in vivo (Figure 6).

5. Discussion

The role of platelets in hematogenous metastasis has been recognized for many years (review in [6,23]). Indeed, platelet-deficient mice as well as mice with mutations rendering platelet non-functional, demonstrated decreased metastases from lung carcinoma and melanoma cell injections [24–26]. A number of mechanisms have been proposed to explain this intervention of platelets. They can protect tumor cells from the immune system, mainly by preventing Natural Killer cells-induced lysis [7]. They can also stimulate tumor growth and influence angiogenesis by releasing a number of molecules stored in their granules [27,28]. Also, adhesion of tumor cells to platelets can lead to the formation of micro-thrombi which facilitate the metastatic process by allowing...
tumor cells to arrest in the blood stream and to adhere to the vascular wall. In order for tumor cells and platelets to interact, the intervention of adhesive proteins capable of bridging these cells together is required. VWF is a good candidate in this respect. Indeed its multimeric structure enables it to interact with more than one ligand at one time and receptors for VWF are present both on platelets and on tumor cells. We have previously described and characterized an in vitro interaction between VWF and murine melanoma cells B16-BL6, interaction involving the Arg-Gly-Asp sequence on VWF and the αvβ3 integrin on tumor cells [13]. We therefore decided to test the role of VWF in an experimental metastasis model in vivo. Surprisingly we observed a significantly increased number of pulmonary metastasis in the absence of VWF when melanoma B16-BL6 cells were injected intravenously. In addition, similar results were obtained with a second tumor cell line, the LLC cells (Figure 1). No significant difference was detected in the size or distribution of the metastatic foci that formed in control and VWF-deficient mice. This suggests that it is the initial establishment of metastatic foci and not their growth that is affected in the knockout mice. Previous studies in mice had reported that antibody-mediated inhibition of VWF led to an inhibition of metastasis formation [20]. It is not the first time that opposite results seem to arise from genetically-altered mice compared to inhibition studies with pharmacological agents. A pro versus anti-angiogenic role has been attributed to αv integrins when antibodies or genetically-deficient mice were used, respectively [29]. For VWF, a major difference between the two studies also comes from the total absence of VWF in the VWF-deficient mice while in the antibody study, only the VWF plasma compartment is targeted.

Our results raise the questions of whether VWF plays a protective role against tumor metastasis through a direct or an indirect effect caused by the mistargeting of proteins normally present in the Weibel-Palade bodies. Since these storage granules do not form in the absence of VWF [30], the altered trafficking of proteins such as P-selectin, endothelin, osteoprotegerin and angiopoietin-2 among others may affect the metastasis process in VWF-deficient mice. To rule out this possibility, we restored VWF plasma concentration in VWF-deficient mice by co-injecting the B16-BL6 cells with purified VWF. This led to a correction of the phenotype, establishing the direct protective role of VWF in metastasis.

We next performed a series of in vitro tests to study how VWF could affect tumor cells behavior. We established that VWF does not affect tumor cells proliferation, migration or invasion through a basement membrane (Figures 2, 3). However, in a cellular death assay, VWF clearly induced death of B16-BL6 cells (Figure 4). Preliminary experiments suggest that VWF induce tumor cells to enter apoptosis but more experiments need to be done to confirm this finding. Whether VWF can also induce tumor cell death in vivo was studied by following the fate of radiolabeled tumor cells during the 24h following their injection in mice. In both WT and VWF-deficient mice, B16-BL6 cells disappeared very quickly from the circulation, indicating no difference in extravasation or in their capacity to form intravascular tumors [31]. This finding is in agreement with the in vitro data showing no effect of VWF on tumor cell invasion. The initial arrest of tumor cells in the lungs was also not influenced by VWF as there was no difference in the percentage of cells present in the lungs of mice of either genotype after 15 min and 1 h (Figure 6). However, at later time-points (4 and 24h), the number of viable cells was greater in the lungs of VWF-deficient mice, suggesting that VWF can induce the death of tumor cells in the hours following their arrest in the lungs and confirming the in vitro data.

This newly described protective role of VWF in tumor metastasis is of particular interest in light of previous studies describing a link between tumorigenesis and a number of molecules essential for blood coagulation and normal platelet function. These include thrombin, tissue factor, P-selectin, Factor VIII, fibrinogen and very recently the platelet VWF receptor GPIbα [24,32–36]. Inhibition of any of these molecules usually leads to a reduction of metastasis, confirming that coagulation and platelet function support tumor metastasis. The pro-metastasis effect obtained by inhibition of VWF is therefore quite unique in this respect. However, the observation that the functional absence of GPIbα results in reduced experimental metastasis, seems to suggest that the mechanism by which VWF inhibits metastasis may be independent of its function in coagulation. Indeed our preliminary results indicate that the capacity of VWF to directly bind tumor cells induces cell death. Further studies are required to confirm and to improve our knowledge of this phenomenon.

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