Deleted in Malignant Brain Tumors 1 is up-regulated in bacterial endocarditis and binds to components of vegetations

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Objective: Bacterial endocarditis is a frequent infectious cardiac disease, especially in patients with congenital or acquired heart defects. It is characterized by bacterial colonization of the heart valves and the appearance of vegetations consisting of fibrin, blood cells, and bacteria. The glycoprotein Deleted in Malignant Brain Tumors 1 is a scavenger receptor cysteine-rich protein with functions in innate immunity and epithelial differentiation. Because of the aggregating capacity of Deleted in Malignant Brain Tumors 1, we hypothesized that an up-regulation in bacterial endocarditis may be linked to the development of vegetations.

Methods: Heart tissue of 19 patients with bacterial endocarditis and 10 controls without bacterial endocarditis was analyzed by immunohistochemistry. The effect of human recombinant Deleted in Malignant Brain Tumors 1 on erythrocyte aggregation was measured using an automated red blood cell aggregometer MA1. Binding of human recombinant Deleted in Malignant Brain Tumors 1 to erythrocyte membranes, platelets, fibrin, and fibrinogen was analyzed by Western blotting and enzyme-linked immunosorbent assay.

Results: Deleted in Malignant Brain Tumors 1 expression was up-regulated in affected heart valves with bacterial endocarditis and limited to the colonizing bacteria on the heart valves and granulocyte-depleted fibrin/fibrinogen formations, and around localized atheromatosis. Patients with aggressive bacteria showed higher DMBT1 levels than patients with less aggressive bacteria. Human recombinant Deleted in Malignant Brain Tumors 1 aggregates erythrocytes and binds to erythrocyte membranes, platelets, and fibrin/fibrinogen.

Conclusion: Deleted in Malignant Brain Tumors 1 up-regulation at sites of bacterial endocarditis, its association with platelets and fibrin/fibrinogen, and its ability to aggregate erythrocytes through binding to their membranes indicate a potential role in the development of vegetations and thrombosis.

Bacterial endocarditis includes infection of the heart valves caused by intravascular bacteria, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus sanguis*, and is often accompanied by fibrin formation on endocardial tissue.^{1,2} Whereas bacterial endocarditis caused by *S aureus* infection often leads to an acute and massive destruction of either previously intact heart valves or heart valves with

congenital or other structural abnormalities, *S epidermidis* and viridans streptococci such as *S sanguis* commonly induce bacterial endocarditis in patients with already damaged heart valves or with prosthetic cardiac devices in a more prolonged subacute clinical course.¹⁻⁴ The formation of vegetations at the site of infection is a central process in the early pathogenesis of bacterial endocarditis. Simplified, these vegetations mainly consist of fibrin, platelets, and the infecting bacteria.¹ Patients with bacterial endocarditis can have embolism of part of the vegetations, leading to infectious insults in different other organs. Another common and life-threatening complication is sepsis. Fibrin thrombi have been observed in the microvasculature of organs in patients with bacterial sepsis and concomitant multisystem organ failure, because sepsis alters the procoagulant–anticoagulant balance with a decrease in anticoagulant factors and an increase in procoagulant factors. Cytokines activate endothelial cells to up-regulate adhesion receptors, which culminates in additional binding of neutrophils, monocytes, macrophages, and platelets to the endothelial cells enhancing the pathophysiologic condition.⁵

Deleted in Malignant Brain Tumors 1 (DMBT1), also known as glycoprotein-340 or salivary agglutinin, is a secreted protein that belongs to the scavenger receptor cysteine-rich group B protein family.⁶ DMBT1 has been proposed to play a role in epithelial differentiation and innate pathogen defense by acting as a pattern recognition

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Abbreviations and Acronyms

ACD	=	acidic citrate dextrose
DMBT1	=	Deleted in Malignant Brain Tumors 1
hr	=	human recombinant
HRP	=	horseradish peroxidase
Ig	=	immunoglobulin
PBS	=	phosphate-buffered saline

molecule.⁷⁻¹⁰ Secreted and membrane-linked pattern recognition molecules share the property to interact with a broad range of pathogens through recognition of repeated molecular patterns commonly located on the pathogen surface. Accordingly, DMBT1 is most prominently expressed at sites with frequent pathogen contacts, such as the respiratory and gastrointestinal epithelium.^{6,7,9}

DMBT1 interacts with surfactant proteins A and D,^{7,11,12} as well as with secretory immunoglobulin (Ig)A,¹³⁻¹⁵ activates the complement system, and may stimulate alveolar macrophages.^{11,16} Furthermore, DMBT1 is up-regulated during infections in infants and adults.^{17,18} In particular, DMBT1 has been demonstrated to bind and aggregate a broad range of bacteria, including Staphylococci and Streptococci, which includes recognition of poly-phosphorylated surface patterns (ie, lipopolysaccharide and lipoteichoic acid).¹⁹⁻²² To this end, in vitro studies suggest that DMBT1 interaction with pathogens is sufficient to efficiently suppress viral and bacterial infection.²³⁻²⁵ However, as recently reviewed, its ability to bind and aggregate pathogens and other endogenous interaction partners may also exert disease-promoting effects in some scenarios.²⁶ In the present study, we analyzed DMBT1 expression in situ in bacterial endocarditis and its interaction with platelets, erythrocytes, and fibrin/fibrinogen, which represent the main constituents of endocarditis-associated vegetations and thrombi.

MATERIALS AND METHODS

Antibodies Used for Immunohistochemistry, Western Blotting, Immunoblotting, and Enzyme-Linked Immunosorbent Assay

For immunohistochemistry, we used the polyclonal anti-serum (anti-DMBT1p84) generated against the recombinant DMBT1 in rabbit. Its specificity was confirmed by a comparison of immunohistochemical sections with RNA in situ hybridization (data not shown). Western blots and enzyme-linked immunosorbent assays were performed with the DMBT1-specific monoclonal antibody Hyb213-06 (Antibodyshop, Gentofte, Denmark) generated against DMBT1^{GP340}, which was isolated from bronchoalveolar lavage by Holmskov and coworkers.¹² The specificity of this antibody was confirmed by Western blotting.²⁷

Immunohistochemical Analysis

We studied the cardiac tissue of 19 adults with bacterial endocarditis and 10 adults without endocarditis as controls (Table 1). The paraffin-embedded sections were examined in cooperation with the Institute of Pathology, University of Heidelberg, Germany. Tissue displaying no signal was scored

0. DMBT1-positive tissue was scored semiquantitatively from 1 (weak staining) to 3 (highly intensive staining). The study was approved by the responsible ethics committee.

An automated Ventana Discovery stainer (Ventana Medical Systems, Tucson, Ariz) was used for immunohistochemical studies with anti-DMBT1p84. The anti-DMBT1p84 antibody was diluted 1:100 in Discovery Ab Diluent, and sections were incubated for 40 minutes, followed by an incubation for 30 minutes with mouse anti-rabbit IgG-AP (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1:500. Peroxidase activity was detected with 3-amino-9-ethylcarbazole as substrate (Sigma Chemicals, St Louis, Mo). Negative control sections were stained with equal amounts of normal rabbit IgG (Santa Cruz Biotechnology) to rule out unspecific binding.

Production and Purification of Human Recombinant Deleted in Malignant Brain Tumors 1

Human recombinant (hr)DMBT1 was produced and purified as described elsewhere.²⁸ A concentration of 10 µg/mL hrDMBT1 corresponds to approximately 40 nmol/L.

Erythrocyte Aggregation

Venous blood was collected from 8 healthy male volunteers of hospital staff after informed consent. Blood samples were anticoagulated with EDTA and lithium heparin to study the influence of calcium ions. Analysis of the effects of calcium in EDTA-treated blood was not possible, because EDTA completely binds calcium ions. EDTA was also added to lithium heparinized blood to complex the calcium ions.

hrDMBT1 was added to the blood samples at final concentrations of 16 to 48 nmol/L. Because hrDMBT1 was dissolved in phosphate-buffered saline (PBS), we used blood samples with equivalent volumes of PBS as controls. The hematocrit was adjusted to 40% by adding plasma of the same person to the blood sample. Erythrocyte aggregation was measured using an automated red blood cell aggregometer MA1 (Myrenne GmbH, Roetgen, Germany). After shearing at 600/s to disperse preexisting aggregates, the erythrocyte aggregation of 20 µL blood sample at stasis (M) was analyzed by integrating light transmission through the sample. The experiments were performed at room temperature within 4 hours.

Incubation of Erythrocyte Membranes With Human Recombinant Deleted in Malignant Brain Tumors 1 and Western Blotting

Lithium heparin blood (7.5 mL) was washed with 0.9% sodium chloride followed by centrifugation at 3000 rpm for 10 minutes and removal of the supernatant. This washing procedure was performed 3 times. After incubation of the erythrocytes with lysis buffer (4.15 g NH₄Cl, 0.5 g KHCO₃, 0.02 g EDTA (Merck, Darmstadt, Germany) in 500 mL aqua; pH 7.37), the lysed cells were centrifuged at 30,000g for 10 minutes and the supernatant was removed. This erythrocyte lysis was performed 3 times. The pellets containing the erythrocyte membranes were incubated with hrDMBT1 (solved in PBS; final concentration 200 nmol/L) or PBS for 30 minutes, followed by washing with PBS. Then the pellets were solved in 60 µL PBS as samples for Western blotting. The samples were separated under nonreducing conditions on 7.5% polyacrylamide gels (Tris-HCl; Bio-Rad Laboratories, Munich, Germany) followed by transfer onto a nitrocellulose membrane. The membrane was blocked with 10% (w/v) milk powder in PBS. For detection of DMBT1, we used the monoclonal antibody Hyb213-06 (1:1500–2000; AntibodyShop). A normal mouse IgG antibody (Santa Cruz Biotechnology) in the same concentration served as the control. As secondary antibody, a sheep anti-mouse IgG-horseradish peroxidase (HRP) conjugate (1:1000; Chemicon, Temecula, Calif) was used. The membranes were developed with an enhanced chemiluminescence kit (ECL Western Blotting Detection, Amersham, GE Healthcare, Braunschweig, Germany) according to the protocols provided by the supplier.

TABLE 1. Clinical parameters and staining scores of controls and cases

No.	Age (y)	Sex	Heart tissue	Score	Bacteria
1	36	M	Aortic valve	0.5	Control
2	78	F	Aortic valve	0	Control
3	61	M	Mitral valve	0.5	Control
4	72	F	Aortic valve	1	Control
5	79	F	Mitral valve	0.5	Control
6	62	M	Aortic valve	1	Control
7	72	F	Aortic valve	0	Control
8	83	M	Aortic valve	0.5	Control
9	73	M	Aortic valve	0.5	Control
10	54	M	Aortic valve	0.5	Control
11	48	M	Tricuspid valve (ulcerous-polypous) Mitral valve (ulcerous-polypous)	3	<i>Staphylococcus aureus</i>
12	72	M	Mitral valve (ulcerous-polypous)	2	<i>S aureus</i>
13	72	M	Mitral valve (ulcerous-polypous)	2.5	<i>S aureus</i>
14	78	M	Aortic valve (in part ulcerous)	3	<i>S aureus</i> , <i>Streptococcus pneumoniae</i>
15	75	M	Mitral valve (ulcerous-polypous)	2	MRSA
16	60	M	Aortic valve (ulcerous-polypous)	0.5	MRSA
17	58	M	Aortic valve (ulcerous-polypous)	3	MRSA
18	57	F	Mitral valve (ulcerous-polypous)	3	<i>Escherichia coli</i>
19	35	M	Aortic valve (ulcerous) Pulmonal valve	1.5	<i>Enterococcus faecalis</i>
20	71	M	Mitral valve (ulcerous-polypous) Aortic valve (ulcerous-polypous)	1.5	<i>E faecalis</i>
21	47	M	Aortic valve (ulcerous-polypous)	1.5	<i>E faecalis</i>
22	36	M	Aortic valve (ulcerous-polypous) Mitral valve (ulcerous-polypous)	1.5	<i>S epidermidis</i>
23	36	M	Aortic valve (ulcerous-polypous)	1.5	<i>S viridans</i>
24	39	M	Aortic valve (ulcerous-polypous)	1.5	<i>S viridans</i>
25	52	M	Aortic valve (ulcerous-polypous)	0	<i>S bovis</i>
26	69	F	Aortic valve Tricuspid valve	2	<i>S lugdunensis</i>
27	32	F	Mitral valve (ulcerous-polypous)	1.5	<i>Streptococcus spp</i>
28	45	M	Mitral valve (ulcerous-polypous)	1	<i>Streptococcus spp</i>

TABLE 1. Continued

No.	Age (y)	Sex	Heart tissue	Score	Bacteria
29	38	M	Aortic valve (ulcerous-polypous) Tricuspid valve (in part necrotizing)	2	None (after long therapy with antibiotics)

MRSA, Methicillin-resistant *Staphylococcus aureus*. Bacteria were detected in blood culture and/or in culture of an intraoperative smear of the concerned valves.

Incubation of Platelets With Human Recombinant Deleted in Malignant Brain Tumors 1 and Western Blotting

Five milliliters of acidic citrate dextrose (ACD: 85 mmol/L sodium citrate, 65 mmol/L citric acid, 125 mmol/L dextrose) were added to 45 mL of venous blood of a healthy volunteer followed by centrifugation at 800 rpm for 20 minutes without brake. After gently mixing the platelet-rich plasma with ACD (1 mL ACD per 5 mL platelet-rich plasma), centrifugation at 2100 rpm for 7 minutes with brake was performed. The supernatant was then discarded, and the platelets were resuspended in washing buffer (Hepes tyrode, consisting of 136 mmol/L sodium chloride, 2.68 mmol/L potassium chloride, 0.98 mmol/L magnesium chloride, 3.26 mmol/L NaH₂PO₄, 3.78 mmol/L Hepes, 5.5 mmol/L glucose and adjusted to pH 7.35, and ACD) followed by centrifugation at 2100 rpm for 4 minutes with brake. After removal of the supernatant, the platelets were resuspended in PBS and washed 3 times with PBS. Activation of platelets with 0.2 U/mL thrombin (Haemochrom Diagnostica, Essen, Germany) was performed at 37°C for 10 to 15 minutes in the presence of H-Gly-Pro-Arg-Pro-NH₂ (5 mmol/L; Sigma-Aldrich, Hamburg, Germany) to prevent platelet aggregation. After 3 washes with PBS, the platelets were incubated with a 200 nmol/L solution of hrDMBT1 (hrDMBT1 was solved in PBS) or PBS alone for 60 minutes at room temperature. Then the pellets were washed 3 times with PBS followed by resuspension in PBS as samples for Western blotting (sodium dodecyl-sulfate polyacrylamide gel electrophoresis, 7% acrylamide). Immunoblotting was carried out as described above using the monoclonal antibody Hyb213-06 (1:2500; AntibodyShop) for DMBT1 detection. In this immunoblot we used a goat anti-mouse IgG-HRP conjugate (1:20,000; Calbiochem, Gibbstown, NJ) as a secondary antibody. For comparison of the loaded platelets in each line, we simultaneously used a goat antibody against platelet/endothelial cell adhesion molecule (PECAM1) (1:1000; Santa Cruz Biotechnology) and an anti-goat-IgG-HRP conjugate (1:20,000; Calbiochem) as secondary antibody.

Binding of Human Recombinant Deleted in Malignant Brain Tumors 1 to Fibrin/Fibrinogen

Enzyme-linked immunosorbent assay microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with fibrinogen (>70% clottable; Sigma-Aldrich) in serial 1:2 dilutions in carbonate buffer for 2 hours at room temperature. Fibrin coating was performed similar to the description by Sahni and Francis.²⁹ Briefly, the wells were coated using 30 µg/mL fibrinogen (>70% clottable; Sigma-Aldrich) in PBS to which 1 U/mL thrombin (Haemochrom Diagnostica, Essen, Germany) was added. After mixing, the solution was rapidly distributed to the consecutive wells in dilution steps of 1:2 in carbonate buffer followed by aspiration after 45 seconds and before polymerization. At 37°C, this procedure leads to a thin fibrin coating of the wells. After washing with PBS containing 2 mmol/L CaCl₂, the plates coated with fibrin/fibrinogen were incubated with a 0.4 nmol/L solution of hrDMBT1 for 40 minutes at room temperature. After washing with PBS/2 mmol/L CaCl₂, a DMBT1-specific monoclonal mouse antibody Hyb213-06 (1:5000; Antibodyshop) was added. Subsequently, the wells were incubated with an HRP-conjugated anti-mouse antibody (1:10,000; Calbiochem). The bound enzyme was detected by adding tetramethyl benzidine-substrate solution (125 µg/mL 3,3',5,5'-tetramethyl benzidine

FIGURE 1. Immunohistochemical analysis of DMBT1 in heart tissues of patients without and with bacterial endocarditis. Binding sites for anti-DMBT1p84 are shown as brown staining. A–D, Heart tissue without endocarditis of 1 case with staining score 0 (A, B, no. 7 in Table 1) and 1 case with staining score 1 (C, D, no. 6 in Table 1). E–H, Exemplary heart tissues of patients with endocarditis and less aggressive bacteria, with 1 case having a staining score of 0 (E, F, no. 25 in Table 1) and 1 case having a staining score of 1.5 (G, H, no. 24 in Table 1). I–L, Exemplary cardiac tissue of patients with endocarditis and aggressive bacteria. Both cases (I, J, no. 11 in Table 1 and K, L, no. 18 in Table 1) show a staining score of 3. I, J, Bacteria (thick arrows) and neutrophils (fine arrows) stain purple; DMBT1 is associated with bacteria and neutrophils. M, N (no. 14 in Table 1), Strong signals can be observed around localized atheromatosis. O, P (no. 14 in Table 1), Positive DMBT1 signals are found in granulocyte-depleted fibrin/fibrinogen (lower part), whereas granulocyte-rich fibrin/fibrinogen (upper part) remains negative. HE, Hematoxylin-eosin; DMBT1, deleted in malignant brain tumors 1.

[Fluka, Sigma-Aldrich, Buchs SG, Switzerland] in 0.1 mol/L citrate buffer pH 4.5 with 0.05% [v/v] H₂O₂). After incubation for 20 minutes in the dark, the reaction was stopped by addition of 2 mol/L HCl. The intensity of the dye reaction was measured at 450 nm in an enzyme-linked immunosorbent assay reader (Victor², Wallac, Turku, Finland). The analyses were done in triplets. As control, wells without hrDMBT1 incubation and wells with only carbonate buffer instead of fibrin/fibrinogen with and without hrDMBT1 incubation were used. The extinction values obtained from the controls were subtracted from the extinction values of the respective wells with DMBT1 incubation to normalize against nonspecific signals.

Statistics

All statistical calculations were performed with SAS software, release 9.1.3 (SAS Institute Inc, Cary, NC). Statistical analyses for comparing the scores of DMBT1 expression in cardiac tissue were performed with the Mann-Whitney U test.

A special model was used for analysis of the erythrocyte aggregation data. Because the observations were correlated, data were analyzed by multilevel modeling using the SAS procedure PROC MIXED (SAS Institute Inc). Patients' identification was assumed as a random effect, whereas hrDMBT1 concentration and anticoagulation method were considered as fixed effects. Test results with *P* values less than .05 were regarded as statistically significant.

RESULTS

Deleted in Malignant Brain Tumors 1 Is Prevalent at Sites With Bacterial Endocarditis

DMBT1 was previously shown to interact with bacteria in vitro, which also play a role for bacterial endocarditis, such as *S aureus* and Streptococci.²⁰ Moreover, an up-regulation of DMBT1 expression was observed during

FIGURE 2. Erythrocyte aggregation through hrDMBT1. Erythrocyte aggregation was measured for each individual blood preparation ($n = 2-9$ for the individual data points), and then the relative aggregation compared with the sample without addition of DMBT1 was calculated for each individual to account for interindividual differences. The diagram displays mean values and standard errors of the mean. *Conc.*, Concentration; *Li*, lithium; *hep*, heparin; *HrDMBT1*, human recombinant deleted in malignant brain tumors 1.

inflammation at epithelial sites.^{17,30} To initially address the role of DMBT1 in cardiac infections, we first studied heart tissues from 10 patients without bacterial endocarditis and 19 patients with bacterial endocarditis by immunohistochemistry using DMBT1-specific antibodies. Eight of the 19 patients with endocarditis showed colonization with aggressive bacteria (*S aureus*, *Escherichia coli*), and 8 of the 19 patients displayed colonization with less-aggressive bacteria (*Enterococcus faecalis*, *S epidermidis*, *S viridans*, *S bovis*, *S lugdunensis*). This classification is provisional, because some bacteria may be reclassified in the future. There are, for example, some reports that the coagulase-negative bacterium *S lugdunensis* can also induce a very aggressive course.³¹ In 2 patients with endocarditis, *Streptococcus* species were found without the possibility of further characterization, and in 1 patient bacteria were not detectable after long-term therapy with antibiotics (Table 1). In heart tissues of adults without (Figure 1, A–D) and with (Figure 1, E–P) bacterial endocarditis, signals could occasionally be detected in parts of the connective tissue, some endothelia, and in part of the myocytes, whereas no signals were observed in the corresponding negative controls (data not shown). Adult heart tissues with bacterial endocarditis revealed strong signals associated with bacteria colonizing the inflamed valves (Figure 1, I, J) and around localized atheromatosis (Figure 1, M, N). Moreover, positive staining was observed for granulocyte-depleted fibrin/fibrinogen formations, whereas granulocyte-rich fibrin/fibrinogen formations were negative (Figure 1, O, P). The staining scores for the individual cases are depicted in Table 1. Statistical analysis revealed a significant difference between the DMBT1 score of patients with (median: 1.5, range: 0–3) and without (median: 0.5, range: 0–1) endocarditis ($P = .0002$). Further, patients with less aggressive bacteria showed lower DMBT1 levels (median: 1.5, range: 0–2) than patients with

aggressive bacteria (median: 2.75, range: 0.5–3) ($P = .010$). The DMBT1 levels in patients with less aggressive bacteria and patients with aggressive bacteria were significantly elevated compared with the controls ($P = .0048$ and $P = .0015$, respectively). Thus, in bacterial endocarditis DMBT1 was found in association with bacteria, as well as with atheromatosis and fibrin/fibrinogen formations, and its levels correlated with the aggressiveness of the bacteria.

Deleted in Malignant Brain Tumors 1 Increases Erythrocyte Aggregation In Vitro, Which Depends on Plasma Components

DMBT1 interaction with bacteria commonly results in bacterial aggregation, which consecutively may suppress bacterial infection.^{20,32} However, it has been proposed that interaction with and aggregation of host components could result in disease-promoting effects of DMBT1.²⁶ On the basis of the prevalence of DMBT1 in fibrin/fibrinogen formations, we next explored the effect of hrDMBT1 on erythrocyte interaction and aggregation. To avoid interfering effects, the experiments were performed using different anticoagulation agents (EDTA, lithium heparin, and lithium heparin + EDTA). To normalize against interindividual differences, we expressed the data as relative values compared with the basal aggregation without hrDMBT1 addition.

Statistical analysis showed that upon addition of hrDMBT1, the hrDMBT1 concentration ($P = .0003$) as well as the anticoagulation method ($P < .0001$) significantly influenced the red blood cell aggregation. The data demonstrated that hrDMBT1 promoted red blood cell aggregation, which, however, was inhibited by the presence of EDTA (Figure 2). A detectable increase of erythrocyte aggregation in blood anticoagulated with lithium heparin started at 32 nmol/L ($P < .0001$) and was further augmented with 48 nmol/L hrDMBT1 ($P < .0001$).

Heat inactivation reduced, but did not completely abolish, hrDMBT1-mediated erythrocyte aggregation (Figure 2). In addition, no aggregation took place when purified erythrocytes were used regardless of whether native or heat-inactivated hrDMBT1 was added. This suggests that DMBT1-mediated erythrocyte aggregation may partly depend on the presence of Ca ions and the 3-dimensional structure of the protein. In particular, however, the data indicated that plasma components might be necessary for DMBT1-mediated erythrocyte aggregation.

Deleted in Malignant Brain Tumors 1 Displays Plasma Component-Independent Binding to Erythrocyte Membranes and Platelets In Vitro

To explore whether the putative plasma components are required for aggregation of DMBT1-coated erythrocytes or for binding of DMBT1 to the erythrocytes, we next tested its ability to interact with the erythrocyte surface. HrDMBT1 readily interacted with membrane preparations of

FIGURE 3. A, Analysis of DMBT1 binding to erythrocyte membranes. *Lane 1:* purified hrDMBT1 solved in PBS (positive control); *lane 2:* erythrocyte membranes incubated with hrDMBT1; *lane 3:* erythrocyte membranes without hrDMBT1 incubation (incubation with PBS only); *lane 4:* PBS washing solution extracted after the third washing step after hrDMBT1 incubation of the erythrocyte membranes. *Lane 2* demonstrates binding of hrDMBT1 to erythrocyte membranes after hrDMBT1 incubation, whereas erythrocyte membranes without hrDMBT1 incubation showed no bound DMBT1. B, DMBT1 binding to platelets. *Top:* DMBT1 detection with the DMBT1-specific monoclonal antibody Hyb213-06. *Bottom:* detection of platelet/endothelial cell adhesion molecule (PECAM1) with a platelet/endothelial cell adhesion molecule-specific antibody as a control for equivalent loading amounts of platelets. *Lane 1:* purified hrDMBT1 solved in PBS serving as the positive control for DMBT1 detection; *lane 2:* platelets incubated with hrDMBT1; *lane 3:* platelets without hrDMBT1 incubation (incubation with PBS); *lane 4:* platelets incubated with hrDMBT1 after activation by thrombin in the presence of H-Gly-Pro-Arg-Pro-NH₂ to prevent platelet aggregation; *lane 5:* platelets without hrDMBT1 incubation (incubation with PBS) after activation by thrombin in the presence of H-Gly-Pro-Arg-Pro-NH₂ to prevent platelet aggregation; *lane 6:* PBS washing solution extracted after the third washing step after hrDMBT1 incubation of the platelets (*lane 2*); *lane 7:* PBS washing solution extracted after the third washing step after hrDMBT1 incubation of the activated platelets (*lane 4*). DMBT1 attaches to both nonactivated (*lane 2*) and activated platelets (*lane 4*) with no major differences. C, hrDMBT1 interacts with immobilized fibrinogen in a concentration-dependent manner. *Control:* exper-

iments, in which hrDMBT1 incubation was omitted (negative control). *Error bars* are standard errors of the means. D, hrDMBT1 interacts with immobilized fibrin in a concentration-dependent manner. *Control:* experiments, in which hrDMBT1 incubation was omitted (negative control). *Error bars* are standard errors of the mean. DMBT1, Deleted in malignant brain tumors 1.

erythrocytes in the absence of plasma as demonstrated by Western blotting (Figure 3, A). We next tested binding to purified platelets, because these are more relevant for the formation of vegetations during bacterial endocarditis. In the absence of plasma components, DMBT1 also readily bound to the surface of platelets with no major differences between nonactivated and thrombin-activated platelets (Figure 3, B). Thus, we conclude that DMBT1 interaction with the surface of erythrocytes and platelets does not depend on the presence of soluble plasma components, whereas the aggregation of erythrocytes apparently does.

Deleted in Malignant Brain Tumors 1 Interacts with Fibrin and Fibrinogen In Vitro

The initial immunohistochemical studies indicated strong staining of fibrin/fibrinogen formations, raising the possibility that fibrinogen might represent a plasma component that causes aggregation of DMBT1-coated erythrocytes and/or platelets. We therefore finally explored whether DMBT1 might interact with fibrinogen and/or fibrin. By using serially diluted fibrinogen and fibrin immobilized on enzyme-linked immunosorbent assay plates, we found that hrDMBT1 bound in a concentration-dependent manner to both fibrinogen and fibrin with a stronger binding activity for fibrinogen (Figure 3, C, D).

DISCUSSION

DMBT1 is known to interact with and aggregate bacteria such as Streptococci and Staphylococci.^{14,19-21} We thus hypothesized a relationship to bacterial endocarditis. Initial evidence has accumulated that DMBT1 may represent a protein with ambivalent character, possibly exerting both disease-preventing and promoting effects.²⁶ For example, its ability to aggregate bacteria may exert beneficial effects in terms of suppression of infection and inflammation.^{19,32,33} On the other hand, DMBT1-mediated aggregation of cholesterol may promote gallstone formation, and it has been proposed that some pathogens may use DMBT1 for adhesion to host structures (reviewed in Mollenhauer and colleagues²⁶).

Subacute infective endocarditis may develop through invasion of bacteria in a preexisting thrombus, whereas in acute infective endocarditis the thrombus could be initiated by the invading organism (ie, *S aureus*) or the valvular trauma from intravenous catheters or pacing wires. *S aureus* can invade the endothelial cells and increase the expression of adhesion molecules and the procoagulant activity.^{34,35}

iments, in which hrDMBT1 incubation was omitted (negative control). *Error bars* are standard errors of the means. D, hrDMBT1 interacts with immobilized fibrin in a concentration-dependent manner. *Control:* experiments, in which hrDMBT1 incubation was omitted (negative control). *Error bars* are standard errors of the mean. DMBT1, Deleted in malignant brain tumors 1.

We observed that DMBT1 is strongly deposited in the lesions associated with bacterial endocarditis. Further, we detected higher DMBT1 levels in patients with endocarditis with aggressive bacteria in comparison with patients with endocarditis with less aggressive bacteria. Moreover, our data suggest that DMBT1 is able to interact with the erythrocyte and platelet surfaces as well as with fibrin and fibrinogen, which may play a role in DMBT1-mediated erythrocyte and platelet aggregation.

Although the precise mode of interaction between DMBT1 and fibrinogen/fibrin remains to be elucidated, our data suggest a stronger binding to immobilized fibrinogen than to immobilized fibrin. Binding to fibrinogen reached saturation, whereas the binding curve for fibrin still seems to be in the linear range at the concentrations tested (Figure 3, C, D). Both fibrinogen and fibrin harbor interaction sites such as the RGD motif (fibrinogen) or the β 15–42 region (fibrin), which are important for mediating molecular and cellular interactions.³⁶ The fact that DMBT1 can specifically be found in granulocyte-depleted but not in granulocyte-rich fibrinogen/fibrin formations could indicate that DMBT1 binds to or sterically hinders access to these motifs in fibrinogen and fibrin.

On the basis of these data, one may assume that it is beneficial to keep the DMBT1 levels in the bloodstream at a minimum to avoid clogging of blood components. This could be reflected by the patterns observed for endothelia in the heart tissues analyzed within this study. Occasional positivity of endothelial cells indicates low or no basal secretion of DMBT1 in the bloodstream and inducibility in response to yet to be defined stimuli. Similar observations have been made for various other cell types, such as lung and intestinal epithelial cells, of which the latter have been shown to up-regulate DMBT1 in response to bacterial surface structures, and tumor necrosis factor- α .^{17,18,32,33,37} In particular, both the lung and the gastrointestinal tract likewise represent examples for tissues in which a limitation of DMBT1 levels could be beneficial. In the respiratory tract, DMBT1 is assumed to counteract infections, but on the other hand, it may increase the surface tension of the surfactant to a level, presumably interfering with proper ventilation when exceeding certain thresholds.^{7,25,37} In the gastrointestinal tract, DMBT1 may be anti-inflammatory and prevent bacterial infection and/or access of the endogenous microflora to the epithelial cells.^{32,33} On the other hand, however, mouse *Dmbt1* is the major sulfated glycoprotein in intestinal plugs occurring in the *Cfr* knockout mouse.³⁸

Accordingly, different mechanisms are conceivable by which DMBT1 could be involved in bacterial endocarditis and associated atheromatosis. Accumulation of DMBT1 at the site of the lesion can be promoted by bacterial colonization and/or its association with fibrinogen/fibrin, which in turn could accelerate bacterial adhesion to the thrombus and/or the deposition of platelets. Alternatively, DMBT1

could promote the interaction between bacteria and fibrin or platelets. Being covered by fibrin and platelets, bacteria are shielded from immune cells and defense factors.³⁹ It is tempting to speculate that the binding of DMBT1 to fibrinogen/fibrin, erythrocytes, and platelets might play a role in other cardiovascular diseases such as atherosclerosis and thrombosis, which has not been addressed to date. Immunohistochemical analyses analogous to the present study may represent a starting point to test this hypothesis. Remarkably, however, a similar role has been proposed for surfactant protein-D, which is one of the DMBT1 interaction partners. Surfactant protein-D knockout mice develop significantly reduced atherosclerotic lesions compared with wild-type mice, indicating a proatherogenic role for surfactant protein-D.⁴⁰

CONCLUSIONS

Our data demonstrate an enrichment of DMBT1 in lesions associated with bacterial endocarditis, in particular vegetations, and simultaneously show that DMBT1 has the capacity to aggregate erythrocytes in vitro. Further, DMBT1 binds to erythrocyte and platelet surfaces and interacts with immobilized fibrinogen and fibrin in a concentration-dependent manner. These data indicate a possible role of DMBT1 in the formation of bacterial endocarditis-associated atheromatosis.

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