Thrombosed arteriovenous fistula for hemodialysis access is characterized by a marked inflammatory activity

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Thrombosed arteriovenous fistula for hemodialysis access is characterized by a marked inflammatory activity.

Background. Thrombosis is the dominant cause of failure of arteriovenous fistulas for hemodialysis access. Vascular inflammation, an important pathologic change in various human vascular diseases, may be involved in the thrombotic process of arteriovenous fistulas.

Methods. The inflammatory activities of 23 thrombosed and 13 nonthrombosed stenotic arteriovenous fistulas were compared by investigating the contents of macrophages and lymphocytes, and the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and interleukin-6 (IL-6) using immunohistochemistry method. The expression of matrix metalloproteinase (MMP)-2 and MMP-9, which play important roles in thrombosis of human coronary artery, was also investigated. The immunoreaction results were characterized using a semiquantitative scoring system.

Results. The macrophage and lymphocyte contents of the thrombosed group were abundant, and markedly greater than those of the nonthrombosed group \((P < 0.001\) and \(P = 0.001\), respectively). The infiltration of macrophages and neovascularure were spatially closely correlated. The expressions of VCAM-1, IL-6, and TNF-\(\alpha\), but not ICAM-1, were significantly higher in the thrombosed group \((P = 0.031, P = 0.010, P < 0.001,\) and \(P = 1.000\), respectively). The expression of MMP-2 was not different in either groups \((P = 0.344)\). Differential expression of MMP-9 by macrophages near the vascular lumen, but not those distant from the lumen, was observed in most thrombosed specimens.

Conclusion. This study demonstrated that the thrombosed arteriovenous fistula was characterized by marked inflammation. We hypothesize that the preferential expression of MMP-9 at luminal edge may cause disruption of the anticoagulant endothelial barrier and contribute to luminal thrombosis of arteriovenous fistulas.

Hemodialysis vascular access failure is the dominant cause of morbidity and the major cost of care for end-stage renal disease (ESRD) patients [1–4]. Substantial previous studies demonstrated that in more than 80% of the vascular access failures the cause is vascular thrombosis superimposed on preexisting stenosis at the venous outflow of the arteriovenous fistula [5–9]. Surgical thrombectomy remains the standard treatment for salvage of the thrombosed arteriovenous fistulas. However, the mid- or long-term patency rate after thrombectomy is unsatisfactory [10–13]. Any attempt to improve the efficacy of the treatment requires further understanding of the vascular pathology of thrombosed arteriovenous fistulas.

The importance of inflammation in the development of various vascular diseases in both the general population and the uremic patients is gaining increasing appreciation. Thrombotic events in human coronary artery [14, 15] and deep vein of limbs [16–19] have been shown to be associated with inflammation of affected vessels. In human arteriovenous fistulas, inflammation may also be involved in the process of thrombosis. In the Canadian Hemodialysis Morbidity Study, hypalbuminemia was the strongest predictor of arteriovenous graft thrombosis [20]. It is now well accepted that hypalbuminemia is not only an indicator of malnutrition in uremic patients but also occurs as a result of inflammation [21–24]. These observations indirectly support that inflammation might play some role in the thrombosis of arteriovenous fistulas.

Accordingly, this study compared the inflammatory activity of the venous segment of thrombosed versus nonthrombosed arteriovenous fistulas by examining the infiltration of macrophages and lymphocytes, and the expression of inflammation-associated molecules, including the adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and proinflammatory cytokines, interleukin-6 (IL-6) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)}
using the immunohistochemistry method. Since increased expression of matrix metalloproteinases (MMPs) with subsequent extracellular matrix (ECM) dysregulation and loss of the integrity of atheroma is thought to contribute to thrombus formation in human coronary artery disease [25–30], the expression of MMP-2 and MMP-9 was also investigated.

METHODS

Clinical data

From November 2000 to July 2003, 13 nonthrombosed primary stenotic and 23 thrombosed specimens of efferent veins of Brescia-Cimino fistulas were obtained during surgical revision from 36 hemodialysis patients of Chung Gung Memorial Hospital, Taipei, Taiwan. The clinical characteristics of the patients were summarized in Table 1. All the fistulas investigated were radiocephalic. The thrombosed lesions were obtained from patients who received revision due to acute occlusion resulting from thrombosis. The primary stenotic lesions were collected as the nonthrombosed control from patients who underwent surgical revision due to an increased venous pressure and/or low flow volume during hemodialysis without any previous history of thrombosis or receiving percutaneous transluminal angioplasty. For the thrombosed lesions, the surgical procedures were done within 8 hours after the patients arrived at the emergency department. Thrombectomy was performed routinely before the revision procedure. Specimens retrieved from the polytetrafluoroethylene (PTFE) graft–interposed fistulas were excluded. The clinical diagnosis of thrombosis or stenosis was confirmed by duplex ultrasound in all cases. The specimens were briefly rinsed with normal saline and fixed in 10% formalin immediately after being harvested. The tissues were then embedded in paraffin, processed according to conventional technique, and cut into 5 μm thick sections.

Immunohistochemistry

Serial sections from each specimen were reacted with mouse antihuman CD68 (Neomarkers, Fremont, CA, USA) (1:100 dilution) and mouse antihuman CD3ε (Neomarkers) (1:50 dilution) antibodies to identify the
macrophages and T lymphocytes, and with mouse antihuman α-smooth muscle actin (α-SMA) (Sigma Chemical Co., St. Louis, MO, USA) (1:100 dilution), rabbit antihuman von Willebrand factor (vWF) (Dako, Carpinteria, CA, USA) (1:200 dilution) as the cell markers for smooth muscle cells (SMCs) and endothelial cells, respectively. The adjacent sections were reacted with mouse antihuman ICAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:400 dilution), rabbit antihuman VCAM-1 (Santa Cruz Biotechnology) (1:200 dilution), rabbit antihuman IL-6 (Santa Cruz Biotechnology) (1:200 dilution), and goat antihuman TNF-α (R&D Systems, Minneapolis, MN, USA) (1:100 dilution), mouse antihuman MMP-2 (Neomarkers) (1:200 dilution) and rabbit antihuman MMP-9 (Neomarkers) (1:100 dilution) antibodies.

**Single-labeling immunohistochemistry.** First, sections were deparaffinized with xylene and rehydrated in graded ethanol series. The sections were then microwave-heated for 10 minutes in a 0.01 mol/L citrate buffer solution (pH 6.6). After blocking endogenous peroxidase activity with 3% H2O2, the primary antibodies were applied for 60 minutes at room temperature. A biotinylated antitissue, antirabbit or antigoat secondary antibody was then applied for 30 minutes, followed by 10 minutes incubation with peroxidase-conjugated streptavidin. The slides were exposed to 3,3′-diaminobenzidine (DAB) to obtain a brown reaction product and counterstained with contrast blue.

**Double-labeling immunofluorescence.** Double-labeling immunofluorescence was performed on tissue sections adjacent to the sections used for single staining to determine the cell types that express the adhesion molecules, proinflammatory cytokines, and MMPs. It was also performed to examine the spatial correlation of the infiltrated macrophages and neovascularure. The immunostaining for the first marker was performed as described above except that a cyanine 3.18 (Cy3) (Chemicon, Temecula, CA, USA) (1:250 dilution) or fluorescein isothiocyanate (FITC) (Chemicon) (1:50 dilution)–conjugated secondary antibody was used instead. Then the sections were washed copiously with phosphate-buffered saline (PBS) and exposed to the second primary antibody for 60 minutes. Then a corresponding secondary antibody conjugated to different fluorochrome was applied.

**Confocal laser microscope**

Immunofluorescence-labeled sections were examined by confocal laser scanning microscopy (Leica TCS SP2-MP System, Mannheim, Germany), equipped with an argon and helium/neon laser, and fitted with the appropriate filter blocks for detection of Cy3 and FITC fluorescence. The images were taken using single or simultaneous dual channel scanning and were transformed into projection views using sets of consecutive single optical sections. All specimens were examined within 24 hours after the immunolabeling protocol.

**Characterization of leukocyte content and immunoreaction of vessel wall**

The cross-sectional area of each sample was scored for macrophage and T-lymphocyte contents on a semiquantitative scale ranging from 0 to 3 in which 0 indicated absence of the cell; 1, occasional isolated cells; 2, small focal clusters of the cell; and 3, large foci or diffuse presence of the cell.

The immunoreactions of adhesion molecules, proinflammatory cytokines, and MMP-2 were also characterized by a semiquantitative immunoreaction scoring system, which was designed to value the extent and intensity of immunoreaction. The immunoreaction score was defined as 0, no reaction; 1, the total area of positive reaction involving less than one third of the total cross-sectional area; 2, the total area of positive reaction involving larger than one third but less than two thirds of the total cross sectional area; and 3, the total area of positive reaction involving more than two thirds of the total cross-sectional area. When the intensity of the reaction was weak, the immunoreaction score was downgraded by 1. Since the expression of ICAM-1 was limited to the single-layered luminal or neovascular endothelial cells, the immunoreaction score for ICAM-1 was estimated by dividing the total ICAM-1–positive area to the total vWF–positive area, instead of the total cross-sectional area. Scoring was performed independently by two investigators blinded to the clinical characteristics of the samples.

**Statistical analyses**

Owing to the limited number of observations in each group, nonparametric statistical analysis was conducted. Medians of the immunoreaction scores were compared across groups using the Mann-Whitney test. When comparing continuous data among independent groups, the Mann-Whitney test was used. All P values presented were two-sided and the level of significance was 0.05.

**RESULTS**

**General observation**

The gross histologic findings in both the thrombosed and nonthrombosed group were similar except that some platelet-rich luminal thrombosis was occasionally found in thrombosed specimens but never in the nonthrombosed specimens. In the thrombosed group, a partial loss of the luminal endothelial layer was often found, which might partially be attributed to the thrombectomy procedure performed routinely before resection of the stenotic segments. The intimal layers of all specimens were composed of neointimal tissue, which encroached upon most
of the luminal space. In most specimens of both groups, neovascularization was common in the intimal and media layers.

**Macrophages and lymphocytes infiltration**

Macrophages, identified by positive anti-CD68 reaction, were found in all (100%) the thrombosed specimens. The cell content was abundant and distributed diffusely or in a large cluster at both the neointima and media (Fig. 1A and B). Lymphocytes, identified by positive anti-CD3ε reaction, were found in 17 of the 23 (74%) thrombosed lesions. The cells distributed in a small to large cluster mainly in neointima of the specimens (Fig. 1C and D). In contrast to the thrombosed group, macrophages and lymphocytes were found in only six and four of 13 (46% and 31%) nonthrombosed specimens, respectively (Fig. 1E to H). In these specimens, isolated lymphocytes were found in the intima or media. The scores for macrophage and lymphocyte content were markedly higher in the thrombosed group 3.0 vs. 0.5, \( P < 0.001 \); and 1.5 vs. 0, \( P < 0.001 \), respectively) than in the non-thrombosed group. Of interest is that the area of abundant macrophages infiltration was constantly associated with infiltration of neovasculature at both intima and media. Double immunofluorescence labeling, observed by laser confocal microscope, confirmed the spatial

![Fig. 1. Representative photomicrographs of the thrombosed (A to D) and the nonthrombosed (E to H) arteriovenous fistula for vascular access demonstrate the leukocytes contents. B, D, F, and H show the higher magnification of the area indicated by the inset in A, C, E, and G, respectively. (A and B) Abundant CD68-positive macrophages (arrows) infiltrate into the neointima (N) and media (M) of the thrombosed arteriovenous fistula. (C and D) Several CD3ε-positive lymphocytes (arrows) infiltrate into the neointima of the thrombosed arteriovenous fistula. (E and F) No CD68-positive cell is found in the neointima or media of the nonthrombosed arteriovenous fistula. (G and H) No CD3ε-positive is found in the neointima or media of the nonthrombosed arteriovenous fistula [original magnification (A, C, E, and G) ×100; (B, D, F, and H) ×400].](image1)

![Fig. 2. Representative double-labeling immunoconfocal images of the thrombosed vascular access demonstrate the spatial correlation of the infiltrating macrophages and the neovasculature. (A) A cluster of von Willebrand factor–positive neovasculature (green color, small arrows) is demonstrates at the lower part of the panel; and the luminal endothelial cells (green color, large arrows) at the upper left. (B) Abundant CD68-positive macrophages (red color, arrowheads) gather at the lower part of the panel around the location of the neovasculature shown (A). (C) Superimposed confocal image of higher magnification confirms that infiltration of the macrophages (red color, arrowheads) is of close spatial correlation with the neovasculature (green color, small arrows) [original magnification (A and B) ×200; (C) ×400].](image2)
Fig. 3. Representative photomicrographs of the thrombosed (A, C, and E) and the nonthrombosed (B, D, and F) arteriovenous fistula for vascular access demonstrate expressions of adhesion molecules and proinflammatory cytokines. (A) Strong antivasular adhesion molecule-1 (anti-VCAM-1) immunoreaction is identified at the neovascular endothelial cells, and smooth muscle cells (SMCs) of neointima (N) and media (M) of the thrombosed arteriovenous fistula. (B) In contrast, the anti-VCAM-1 immunoreaction is much weaker in the nonthrombosed specimen localized to the neovascular endothelial cells and some medial SMCs. (C) Anti-intercellular adhesion molecule-1 (anti-ICAM-1) immunoreaction is identified at the neovascular endothelial cells of the thrombosed arteriovenous fistula. (D) Similar anti-ICAM-1 immunoreaction is demonstrated in the nonthrombosed specimen. (E) Strong anti-interleukin-6 (anti-IL-6) immunoreaction is identified at neovascular endothelial cells, medial SMCs, and extracellular matrix (ECM) of the thrombosed specimen. (F) In the nonthrombosed specimen, only a very weak focal anti-IL-6 reaction is shown at the ECM of media of the nonthrombosed specimen. (G) Positive anti-tumor necrosis factor-α (anti-TNF-α) immunoreaction is identified in the neovascular endothelial cells and ECM of the neointima of the thrombosed specimen. (H) No positive anti-TNF-α is detectable in the nonthrombosed specimen (original magnification ×100).

Correlation between infiltration of macrophages and neovascularature (Fig. 2).

Expression of adhesion molecules

Positive immunoreaction for VCAM-1 was found in 20 of the 23 (87%) thrombosed and 10 of the 13 (77%) non-thrombosed specimens. Positive labeling was localized to the luminal and neovascular endothelial cells, and SMCs of neointima and media in both groups if present. The staining for VCAM-1 in the thrombosed group was much more extensive with a stronger intensity in the thrombosed group when compared with the nonthrombosed group (Fig. 3A and B) The immunoreaction score was significantly higher in the thrombosed group (2.5 vs. 1.0, $P = 0.031$). Positive immunoreaction for ICAM-1 was detected homogeneously on luminal and neovascularualr...
endothelial cells with strong intensity in all specimens of both the thrombosed and nonthrombosed groups (Fig. 3C and D). The immunoreaction scores of both groups were similar (3.0 vs. 3.0, \( P = 1.000 \)).

**Expression of cytokines**

Positive immunoreaction for IL-6 was found in all the thrombosed (100%) and eight of the 13 (62%) nonthrombosed specimens. Positive staining was localized to the SMCs and ECM of neointima and media, and neovasculature, if present. The immunoreaction was more extensive with a stronger intensity in the thrombosed group when compared with the nonthrombosed group (Fig. 3E and F) The immunoreaction score was significantly higher in the thrombosed group when compared with the nonthrombosed group (2.5 vs. 1.0, \( P = 0.010 \)). Positive immunoreaction for TNF-\( \alpha \) was found in 13 of the 23 (57%) thrombosed specimens and none of the nonthrombosed group. The reaction was distributed in a small or large focus and localized mainly to the ECM of the neointima or media and rarely to the neovasculature (Fig. 3G and H) The immunoreaction score for TNF-\( \alpha \) was significantly higher in the thrombosed group (1.0 vs. 0, \( P < 0.001 \)).

**Expression of MMPs**

Positive immunoreaction for MMP-2 was found in all (100%) of the specimens of both the thrombosed and nonthrombosed groups. The immunostaining was localized to the SMCs, ECM, and neovasculature of the neointima and media. The extension and intensity of positive reaction varied markedly among specimens. The immunoreaction scores were similar in both groups (2.0 in thrombosed group vs. 1.5 in nonthrombosed group) (\( P = 0.344 \)). The expression of MMP-9 was much more limited than that of MMP-2. In the nonthrombosed group, positive reaction was found in only one sample (7%), in which the reaction was localized to few isolated macrophages in the neointima. In the thrombosed group, 21 of the 23 (91%) specimens showed positive immunoreaction to anti-MMP-9. The reaction was localized to few isolated or a small cluster of cells at the neointima near the vascular lumen. Double immunolabeling recognized those MMP-9–positive cells as macrophages. Although macrophages were abundant and widespread at the neointima and media of the thrombosed specimens, most macrophages located distant from the lumen at the deeper neointima layer or the media did not express MMP-9. Imaging by confocal laser microscope confirmed the preferential expression of MMP-9 (Fig. 4).

The scores for macrophage and lymphocyte contents and immunoreaction scores for the adhesion molecules, proinflammatory cytokines, and MMPs are summarized in Table 2.

**DISCUSSION**

This study demonstrated infiltration of abundant macrophages and small to moderate amounts of lymphocytes in the vascular wall of thrombosed arteriovenous fistula accompanied by increased expression of VCAM-1 and proinflammatory cytokines, IL-6 and TNF-\( \alpha \). These findings indicated a marked inflammatory activity involved in the vascular wall of thrombosed arteriovenous fistulas.

Surgical thrombectomy is conventionally performed to treat the thrombosed arteriovenous fistula. Several new techniques have been reported in attempt to remove thrombus percutaneously in recent years. However, the mid- or long-term patency rate after these procedures remains low [10–13]. The marked inflammatory activity of the thrombosed arteriovenous fistula may potentially exert several adverse biologic effects in maintaining the arteriovenous fistula patent. Intracellular IL-6 was shown to stimulate proliferation of vascular SMC in a platelet-derived growth factor (PDGF)–dependent manner [31, 32]. Additionally, VCAM-1 was demonstrated by Oguchi et al [33] to be involved in the activation of vascular SMCs and neointimal formation in apolipoprotein E knockout mice. Increased expression of IL-6 and VCAM-1 in the thrombosed arteriovenous fistula demonstrated in the present study may enhance the vascular SMC proliferation and subsequent progression of neointimal formation in the thrombosed arteriovenous fistula. Therefore, inflammatory activity in the thrombosed arteriovenous fistulas may contribute to the low long-term patency rate after thrombectomy.

In human coronary artery, inflammation is thought to contribute to the luminal thrombosis in acute coronary syndrome [14, 15]. In these events, ulceration or rupture of the atheromatous plaque with subsequent exposure of the highly procoagulant subendothelial tissue to the

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**Table 2.** Scores of leukocyte contents and immunoreaction scores of the thrombosed and nonthrombosed vascular accesses

<table>
<thead>
<tr>
<th></th>
<th>Macrophage</th>
<th>Lymphocyte</th>
<th>VCAM-1</th>
<th>ICAM-1</th>
<th>IL-6</th>
<th>TNF-( \alpha )</th>
<th>MMP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombosed</td>
<td>3.0 (3.0–2.8)</td>
<td>1.5 (0.5–2.0)</td>
<td>2.5 (1.3–3.0)</td>
<td>3.0 (3.0–3.0)</td>
<td>2.5 (1.5–3.0)</td>
<td>1.0 (0–1.5)</td>
<td>2.0 (1.0–2.5)</td>
</tr>
<tr>
<td>Nonthrombosed</td>
<td>0.5 (0–1.0)</td>
<td>0 (0–0.5)</td>
<td>1.0 (0.8–1.8)</td>
<td>3.0 (3.0–3.0)</td>
<td>1.0 (0–2.0)</td>
<td>ND</td>
<td>1.5 (1.0–2.0)</td>
</tr>
<tr>
<td>( P ) value</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>0.031</td>
<td>1.000</td>
<td>0.010</td>
<td>&lt;0.001</td>
<td>0.344</td>
</tr>
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Abbreviations are: VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; TNF-\( \alpha \), tumor necrosis factor-\( \alpha \); MMP-2, matrix metalloproteinase-2; ND, not detectable. Values are medians (interquartile range).
circulating blood leads to thrombosis in the vascular lumens [15]. The linkage between inflammation and thrombus formation was thought to be through the proteolytic effect of MMPs expressed by macrophages infiltrating into the atheroma [25–30]. Dysregulation of the ECM by increased expression of MMPs results in structural instability and rupture or ulceration of the atheroma.

The underlying pathologic change of the thrombosed arteriovenous fistula differs from that of the thrombosed coronary artery. The preexisting lesion in thrombosed arteriovenous fistulas is composed of neointimal hyperplasia rather than atherosclerosis. However, the above mechanism may also be involved in the pathogenesis of thrombosis in the arteriovenous fistula. In the present study, preferential expression of MMP-9 by macrophages near the vascular lumen was demonstrated in the thrombosed specimens, which may potentially cause structural instability of the neointimal layer and subsequent disruption of the overlying endothelial layer. To observe the presence of disintegration of superficial neointima around the focus of MMP-9 expression may provide important information to validate this hypothesis. However, thrombectomy performed routinely for the thrombosed arteriovenous fistula before resection of the stenotic segments in the present study might result in denudation in some specimens, and make this observation infeasible.

One other interesting finding in the present study is the strong spatial correlation between the infiltrating macrophages and neovascularure. Similar findings have been demonstrated in the atherosclerotic plaques of human coronary artery by O’Brien et al. [34]. They found that the intimal neovascularure in human coronary athereoma is strongly associated with increased intimal leukocytes infiltration. In apolipoprotein E-deficient mice, Moulton et al. [35] further demonstrated that the inhibition of plaque neovascularization by angiogenesis inhibitor reduced macrophage accumulation. These findings suggest that the neovascularure is an important route for infiltration of leukocytes into the vascular wall and is a potential target in reducing the inflammation of arteriovenous fistulas.

The clinical implication of this study is that anti-inflammatory treatment may be helpful to the thrombosed arteriovenous fistula in blocking the potential adverse biologic effects of inflammatory mediators on the vessel wall and subsequently improve the longevity of the arteriovenous fistula. Further study to clarify the causal role of inflammation on thrombosis of arteriovenous fistulas is necessary to decide whether anti-inflammation could be a potential strategy for prevention of thrombosis of the hemodialysis vascular access.

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