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Vein Tissue Expression of Matrix Metalloproteinase as Biomarker for Hemodialysis Arteriovenous Fistula Maturation

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Abstract

Failure of arteriovenous fistula (AVF) maturation is attributed to impaired vein remodeling. The purpose of this study is to identify whether vein matrix metalloproteinase (MMP) expression and activity is associated with AVF maturation. Patients with renal insufficiency undergoing surgery had their vein segments harvested and snap-frozen at time of AVF construction. Expression of MMP-2, MMP-9, membrane type-1 MMP (MT1-MMP), tissue inhibitor of metalloproteinases type 2 (TIMP-2), and TIMP-4 were measured using zymography and Western blotting techniques. Of 14 patients enrolled, 9 had successful maturation and 5 had failure of AVF maturation. Significantly higher levels of MT1-MMP (an MMP-2 activator; $P = .01$), TIMP-2 (an MMP-2 inhibitor; $P = .03$), MMP-2 latent ($P = .02$), and MMP-2 total ($P = .03$) were associated with AVF maturation. There was a trend toward higher levels of TIMP-4 in the successful group ($P = .18$). These data demonstrate a positive relationship between MMP-2 expression in veins and AVF maturation. MMP-2 could serve as a potential preoperative marker to predict maturation.

Keywords

vessel remodeling; matrix metalloproteinase; hemodialysis; arteriovenous

Introduction

A majority of patients with end-stage renal failure require hemodialysis, and the arteriovenous fistula (AVF) is the preferred method for access. Arteriovenous are the most durable, resistant to infection and thrombosis. Recognizing the superiority of this access, the National Kidney Foundation recommends an aggressive approach to the creation of AVF.¹ However, 20% to 60% of primary AVF fail to develop into a functioning dialysis access.^{2,3} The failure is either by impaired vein remodeling or by intimal hyperplasia leading to thrombosis.

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Declaration of Conflicting Interests

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Vascular access failure is the most important cause for morbidity, repeat surgery, and hospitalization.⁴ Vascular specialists are left with the onerous task of establishing and maintaining hemodialysis access. Given the dismal results, the biochemical and pathological changes associated with AVF maturation and intimal hyperplasia should be sought. Recent studies have shown an important role of matrix metalloproteinases (MMPs) in the process of AVF maturation.^{5,6} Matrix metalloproteinases belong to a group of zinc-dependent proteases capable of degrading extracellular matrix (ECM) proteins.^{7,8} In particular, MMP-2 is expressed by a variety of cell types and activated by membrane-bound membrane type-1 MMP (MT1-MMP) and is inhibited by tissue inhibitor of metalloproteinases type 2 (TIMP-2).⁹ Similarly, MMP-9 is inhibited by TIMP-4. Because MMP-2 and MMP-9 have been found to have increased expression in the outflow vein tissue, after AVF construction,⁶ MMP expression in vein segments at the time of initial surgery may serve as a biomarker of AVF maturation. The purpose of this study is to determine whether human patient vein MMP expression, particularly MMP-2 and MMP-9, can predict successful AVF maturation.

Methods

Human Study Methods

A prospective evaluation of patients undergoing AVF construction for chronic renal insufficiency was performed under institutional review board approval at the Northern California Veterans Affairs Health Care System. All patients were enrolled and followed at the Sacramento Veterans Affairs Medical Center.

After informed written consent was obtained, a patient history and physical examination was performed and pertinent medical history documented. All patients underwent preoperative vein mapping (without a tourniquet) using duplex ultrasonography, documenting vein diameter, patency, as well as arterial patency of both upper extremities. A radial artery diameter of greater than 0.2 cm and a vein diameter of greater than 0.2 cm at the wrist or greater than 0.3 cm at the antecubital fossa was a requirement for primary AVF. The minimum diameter was also a determinant in the location with which the AVF would be constructed. Both upper extremities were mapped and the most distal vein segment in the nondominant extremity was used if all diameter criteria were met.

The construction of the AVF was performed under local anesthesia with monitored anesthesia care. The arterial and venous segments were dissected per routine surgical care. After an adequate length of vein was free, an end of vein to side of artery was constructed. Excess vein segments prior to arterial-venous anastomosis was sharply divided and placed in a cryovial. The cryovial was snap-frozen in liquid nitrogen and stored in a -80°C for later analysis.

After surgical construction, the patients were followed postoperatively, at 2 weeks, 6 weeks, and as-an-as-needed basis depending upon the patients' impending requirement for hemodialysis access. Interventional procedures were planned as necessary as a part of an intent-to-treat basis. Fistula with velocity findings consistent with stenosis was recommended intervention. Patients were deemed to have a successful hemodialysis access when the AVF was successfully accessed on 3 separate occasions for hemodialysis. If the

hemodialysis access was unable to be accessed or the vein segments were too small for hemodialysis access based on duplex ultrasonography, the patients underwent a subsequent hemodialysis access procedure, or the AVF was found to be thrombosed upon examination, the access procedure was considered a failure. The patients were divided into a successful AVF group and a failed AVF group for protein analysis.

Zymographic Analyses of Matrix Metalloproteinases

Vein segments were evaluated by Gelatin Zymography and Western blotting for levels of latent MMP-2, active MMP-2, MMP-9, TIMP-2, TIMP-4, and MT1-MMP. The levels and activities of MMP-2/-9 were first examined by Zymography using the whole-tissue lysate of previously stored venous segments. Briefly, the vein segments were ground in liquid nitrogen using mortar and pestle, followed by homogenization in 1% Radio-Immunoprecipitation Assay (RIPA) lysis buffer (Thermo Scientific, Illinois) containing protease and phosphatase inhibitor cocktails. The protein concentrations of the tissue lysates were determined with a Bio-Rad detergent-compatible protein assay kit (Bio-Rad, California). After vortex and incubation on ice for 10 minutes, the samples were centrifuged at 14 000 rpm for 5 minutes at 4°C on a HERMLE Z180M centrifuge (Labnet, New Jersey). The resultant supernatants were collected and mixed thoroughly with 2× sample buffer (Invitrogen, California) in nonreducing conditions for zymographic gel. Following the manufacturer's instruction, proteins were separated on a 10% zymogram gel. After incubation in renaturing solution, the gel was incubated overnight at 37°C in zymogram-developing buffer. The zymogram gel was then stained with colloidal blue stain for 6 hours and destained with water for 16 hours. The developed bands were then imaged with an AlphaImager EC (Alpha Innotech, California) and quantified by densitometry using ImageJ software (NIH, Bethesda, Maryland). For Western blots, the samples were mixed with 2× sample buffer with reducing reagent and boiled for 6 minutes. Equal amount of protein sample from each vein fragment was separated on a 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel, transferred to a PVDF membrane (Millipore Corporation, Massachusetts), and blocked overnight in 3% Bovine Serum Albumin, BSA (Fisher Scientific, Pennsylvania) at 4°C. The membrane was then immunoblotted sequentially with primary antibodies targeting MMP-2, MMP-9, TIMP-2, TIMP-4 (Santa Cruz, California), MT1-MMP (Chemicon, Illinois), and their corresponding horseradish peroxidase-conjugated secondary antibodies. β -Tubulin (Santa Cruz) was used as a loading control. The proteins of interest were visualized using ECL Plus (General Electric Healthcare, New Jersey) detection system and Kodak BioMax MR Film on an SRX-101A Medical Film Processor (Konica-Minolta). The films were then scanned and processed with ImageJ software for quantification.

Statistics

Results are reported as mean $\pm$ standard deviation. Microsoft Excel (Redmond, Washington) was used to perform a 2-tailed *t* test to compare groups and a difference was considered significant for a *P* value $<.05$.

Results

Fourteen patients were enrolled. Nine patients (age: 63 ± 7 years) had successful AVF, either a radiocephalic or brachiocephalic fistula. Five patients (age: 64 ± 7 years; $P = .7$) had failure of AVF maturation, a 36.7% failure rate. The average vein map diameter was 0.4 ± 0.1 cm in the successful group and 0.4 ± 0.1 cm in the failed group ($P = 1.0$). Patients with diabetes as the cause for renal failure was an indication for the procedure in 67% in the successful group and 60% in the failed group ($P = .9$). Demographic information are summarized in Table 1. During the immediate follow-up period, no interventional procedures were performed on either group.

Significantly higher MT1-MMP expression (MMP-2 activator) was seen in the vein pieces of patients with successful AVF maturation 0.5 ± 0.1 versus failed 0.3 ± 0.2 ($P = .01$). Matrix metalloproteinase-2 latent levels were higher in the successful group 0.9 ± 0.2 versus the failed group 0.6 ± 0.1 ($P = .02$). Similarly, the total MMP-2 levels were higher in the successful group 0.9 ± 0.3 versus 0.6 ± 0.1 ($P = .03$). Increased TIMP-2 expression was found in the successful group 1.7 ± 0.9 versus the failed group 0.6 ± 0.5 ($P = .03$). A trend toward significantly higher levels of TIMP-4 in the successful group was observed 0.6 ± 0.6 compared to the failed group 0.2 ± 0.2 ($P = .2$). No differences were seen in the MMP-2 active ($P = .6$) and the MMP-9 levels ($P = .9$). The data are summarized in Figures 1 and 2.

In 1 patient, the AVF Cimino failed to mature after 3 months of observation and after 4 months, the AVF Cimino thrombosed. A subsequent AVF, a brachiocephalic fistula, was then constructed. The brachiocephalic fistula was successful in maturation. A protein analysis was performed of the vein pieces harvested. Similar to the 14 vein piece vein segment comparisons, MT1-MMP, MMP-2 latent, and MMP-2 total levels demonstrate higher levels in the matured vein piece obtained when constructing the brachiocephalic fistula (Figure 3).

An analysis of the vein segments was also performed after removing this 1 patient's vein segments for analysis. There was no change in the results regarding protein activity in correlating successful AVF maturation with MMP activity.

Discussion

After AVF construction, the outflow vein immediately encounters increased blood flow volume, blood pressure, and blood oxygen content. The fistular outflow vein responds by dilating and wall thickening to sustain blood volume of 300 to 600 mL/min, sufficient for hemodialysis access. At times, the fistular vein may remain patent but fails to undergo the appropriate remodeling termed "fistula maturation." However, the fistular vein may appropriately mature but develop intimal hyperplasia, vein stenosis, and subsequent thrombosis. Through either mechanism, the access has failed.

Given the high rate with which AVF fail to mature, an evaluation of biomarkers would serve as a potential method to select more appropriate procedures and surveillance protocols.

Biomarkers have demonstrated a great value in the diagnosis and treatment of many cardiovascular disease entities such as troponin in the diagnosis of acute myocardial

infarction¹⁰ and C-reactive protein in predicting cardiac risk.¹¹ More recently, circulating biomarkers of fibrinogen, D-dimer, and interleukin-6 (IL-6) have been suggested as areas of interest in detecting the presence and progression of abdominal aortic aneurysms¹² and other types of vascular injury.¹³ Circulating biomarkers offer diagnostic or prognostic value by reflecting the disease state and predilection based on plasma measurements of molecules or proteins.¹¹

Serum biomarkers have been evaluated in renal failure patients with AVF, but there are limitations. C-Reactive Protein (CRP)¹⁴ and serum lipid profiles¹⁵ have been proposed as measures to maintain AVF patency. However, these circulating biomarkers do not address the failure to achieve the initial long-term AVF access site. Serum biomarkers also fail to explain the differential effects frequently seen when a radiocephalic fistula would fail but a subsequent brachiocephalic fistula would mature.

Since the National Kidney Foundation introduced the Dialysis Outcomes and Quality Initiative, guidelines and access management criteria are being used to judge vascular access programs.¹ However, in a recent review of the natural history of autogenous fistulas for first-time dialysis access, only 11% of primary AVF matured without intervention and a total of 48% of the primary AVF was ultimately used for hemodialysis access. The mean time to AVF maturation was 146 days and the mean time for AVF abandonment was 162 days.¹⁶ Given these dismal results, many patients undergo repeat operations and procedures are many times unsuccessful.

To improve on this dismal clinical problem, investigators have focused on vascular remodeling and vascular pathology. In previous studies, the animal model is used to evaluate MMPs which would be an obvious choice as these proteins have played a well-established role in both intimal hyperplasia¹⁷ and vessel enlargement^{5,6} when it comes to AVF maturation. To date, this is the first evaluation of human vein tissue prior to AVF construction.

Misra and associates have found that there is an increased expression in both latent and active MMP-2 activity and increased expression of MMP-9 activity in areas of venous stenosis at an AVF in the rat.¹⁷ Similarly, Chan and associates found that both MMP-2 and MMP-9 expression were increased but that TIMP-4 was downregulated in the venous segments after AVF construction.⁶ These data are important in establishing the role of MMP-2 and MMP-9 upregulation as well as the counterpart inhibitor TIMP downregulation in venous dilation and wall thickening. However, several limitations exist regarding the use of the models described. The animal models have normal renal function that eliminates many factors unique to renal failure patients, such as increased inflammation,¹⁸ uremia, platelet dysfunction, and arterial calcification.¹⁹ Furthermore, the vessels evaluated in the animal models are deep vessels such as the iliac vessels, the carotid artery, and internal jugular veins. Whether the venous changes in constructing a fistula can translate easily to the superficial arterial and venous systems are unknown.

The clinical impact of using preoperative markers resides with its use in counseling patients regarding the presence of appropriate vein segments for potentially successful AVF

maturation. Different cephalic vein MMP activity levels at the wrist and elbow were observed in 1 patient who had a failed radiocephalic fistula and a subsequent mature brachiocephalic fistula. The expression levels mimicked the differences seen between the different patient groups with failed and successful AVF maturation.

Since the Fistula First Initiative, AVF prevalence has increased from 24% to 47% in the United States in 2007. However, central vein catheter use for implementation of dialysis has risen 1.5- to 3.0-fold during that same time period.²⁰ With the use of vein tissue biomarkers, earlier recognition of an AVF that will never mature could reduce patient-catheter days. Rather, earlier intervention to another AVF could be constructed or a primary AV bridge graft could be recommended to the patient.

Misra and associates evaluated vein segments in patients who have had failure of their AVF or hemodialysis access grafts and harvested vein segments distal to the anastomoses.²¹ Latent MMP-2 and latent MMP-9 expression were noted to be higher in failing vein segments secondary to intimal hyperplasia than in the control veins of patients. This is consistent with data regarding increased MMP-2 and MMP-9 activity in intimal hyperplasia but contradicts the role of MMP-2 and MMP-9 activity in appropriate venous enlargement and wall thickening for appropriate AVF maturation.

In this study, vein segments were harvested prior to AVF. If vein diameter expansion were the primary necessary step toward AVF maturation, MMP-2 and MMP-9 activities would be expected to be higher. However, increased MMP-2 and MMP-9 activities should be initially lower in AVF maturation if intimal hyperplasia plays a bigger role in primary AVF failure. The contradictory role of MMP-2 and MMP-9 activity^{3,22} in appropriate AVF may be related to different time courses of the remodeling response.⁵ In early arterial adaptation to high flow rates, a transient increase in MMP-2 activity is found and returns to baseline by 14 days. MMP-9 activity, however, was not found to have a notable increase early after AVF formation. The concept of temporal effects may better resolve the conflicting results of MMP's role in intimal hyperplasia versus vein dilation after AVF construction.

Within this study, patients who had subsequently mature AVF had significantly higher levels of MT1-MMP, TIMP-2, MMP-2 latent, and MMP-2 total within the vein tissues. MMP-2 may play a more important role in the initial vein remodeling after AVF construction and that basal level of MMP-2 is the initial marker necessary to predict AVF maturation. For successful maturation, a balance of MMP-2 and the associated activator MT1-MMP and the inhibitor TIMP-2 are crucial to appropriate vascular remodeling. Too little MMP-2 protein levels will lead to primary failure. Too much MMP-2 will lead to intimal hyperplasia. Larger levels and activity in vein tissue portend appropriate remodeling.

Further differential studies will be performed on vein segments harvested from the cephalic veins at the wrist and elbow as well as comparing the cephalic and the basilic veins at the antecubital region.

Conclusions

A promising biomarker identified in human vein tissue, MMP-2, may serve to predict AVF maturation. Further studies are needed compare the relative importance of other MMP isoforms in the process of AVF maturation.

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Figure 1.

Increased expression of matrix metalloproteinase (MMP)-2 latent, MMP-2 active, MMP-2 total, and tissue inhibitor of metalloproteinases type 2 (TIMP-2) in the vein pieces taken from patients who had arteriovenous fistula (AVF) that matured. *Statistically significant at $P < .05$.

Figure 2.

Increased expression of membrane type-1 matrix metalloproteinase (MT1-MMP), an MMP-2 activator in vein tissues obtained from patients who had arteriovenous fistula (AVF) that matured. Increased expression in the tissue inhibitor of metalloproteinases type 4 (TIMP-4) in the vein tissues obtained from patients with AVF that matured. Due to a large standard deviation, TIMP-4 did not have statistically significant higher expression in the mature group ($P = .18$). Given higher expression of TIMP-4 and appropriate decrease in MMP-9 expression is seen in the mature group, although not statistically different.

*Statistically significant at $P < .05$.

Figure 3.

Vein tissue segments were obtained from 1 patient at the radiocephalic location (failed) and the brachiocephalic location (mature). Representative bar graph demonstrates matrix metalloproteinase (MMP)-2 latent, active, total, membrane type-1 matrix metalloproteinase (MT1-MMP), tissue inhibitor of metalloproteinases type 4 (TIMP-4), and MMP-9 expression. The white bars represent the expression of protein levels in the vein tissue obtained from the failed arteriovenous fistula (AVF), and the black bars represent the expression of protein levels in the vein tissue obtained from the mature AVF. Note that the bar graphs mimic the bar graphs for the entire patient group under investigation.

Table 1
Demographic Description of Renal Failure Patients and Arteriovenous Fistula (AVF) Outcome

Patient	Age	Diagnosis	Vein Diameter	Location	Outcome
1	76	Diabetes	0.4	R elbow	Failed
2	57	Hypertension	0.5	L elbow	Failed
3	64	Diabetes	0.5	R wrist	Failed
4	60	Drug induced	0.2	L WRIST	Failed
5	65	Diabetes	0.4	L elbow	Failed
Average:	64.4 ± 7		0.41 ± 0.1		
6	75	Diabetes	0.4	L wrist	Mature
7	61	Diabetes	0.3	L wrist	Mature
8	54	Diabetes	0.4	L elbow	Mature
9	74	Hypertension	0.4	L elbow	Mature
10	63	Hypertension	0.3	L elbow	Mature
11	58	Diabetes	0.6	R elbow	Mature
12	58	Diabetes	0.3	L elbow	Mature
13	60	Hypertension	0.4	L elbow	Mature
14	64	Diabetes	0.5	R elbow	Mature
Average:	63 ± 7		.041 ± 0.11		