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Short Communication

Down-Regulation of Endothelial Expression of Endothelial Cell Protein C Receptor and Thrombomodulin in Coronary Atherosclerosis

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Coronary atherosclerosis with occlusive thrombosis is the major cause of acute myocardial infarction. Although plaque rupture is usually hypothesized to be the predisposing event in coronary thrombosis, the possibility cannot be excluded that local changes in the anticoagulant properties of the endothelium overlying the plaque contribute to this process. It is evident that thrombomodulin and the endothelial cell protein C receptor are critical players in the control of the thrombogenic process. This study examined whether thrombomodulin and the endothelial cell protein C receptor are down-regulated on endothelial cells overlying the atherosclerotic plaque in coronary arteries and thus could potentially favor local thrombus formation. Sections of archival left and right coronary arteries (n = 18 each) with severe atherosclerosis from the native heart of six patients who underwent heart transplantation were immunostained for CD31, CD34, endothelial cell protein C receptor, and thrombomodulin using a streptavidin-biotin-peroxidase method. Controls included left and right coronary arteries from autopsy cases with no atherosclerosis (n = 6), and also from cases with mild atherosclerosis (n = 5). The apparent density of all of these proteins was much higher in control than in atherosclerotic arteries. Our findings support the hypothesis that both endothelial cell protein C receptor and thrombomodulin are down-regulated in coronary arteries with atherosclerosis. These changes would be expected to result in reduced inhibition of thrombogenic and anti-inflammatory activity on the endothelium overlying atherosclerotic regions and thus could contribute to coronary thrombosis. *(Am J Pathol 2001, 159:797–802)*

Rupture of vulnerable atherosclerotic plaques or erosion of fibrous plaques are the hypothesized predisposing events for coronary artery thrombosis.[1] Thrombosis can result in unstable angina, myocardial infarction, or sudden death. If thrombus formation is limited, the consequences may be less severe or may remain clinically silent. There is a general acceptance that tissue factor exposed and/or released from ruptured plaques plays a major role in initiating thrombosis. However, additional local factors such as altered endothelial anticoagulant properties, blood flow rate, and activity of the fibrinolytic system may also be important in thrombogenesis, ie, influencing the development/severity of thrombosis and therefore, the severity of the consequences of thrombosis.

Thrombomodulin (TM) and the endothelial cell protein C receptor (EPCR) are known critically important molecules in control of the protein C anticoagulant pathway. The physiological importance of these endothelial cell receptors is demonstrated by the observation that gene disruption of either results in early embryonic lethality in mice.[2,3] Maximal rates of protein C activation require thrombin binding to TM as well as protein C binding to EPCR.[4] The formation of the thrombin-TM complex acts as a molecular switch to limit the thrombus-generating function of thrombin and to prevent cellular activation via protease-activated receptors including activation of the endothelium. Activated protein C and TM have been shown to exhibit anti-inflammatory properties.[4] Of particular relevance to the situation in the coronary artery, TM overexpression has been shown to reduce thrombus formation, neointima formation, and macrophage and neutro-

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Table 1. The Cause of Death in the Control Cases

Group 1	Group 2
1. Pulmonary emboli after hip surgery (61/M)	1. Cirrhosis of the liver and bronchopneumonia (40/M)
2. Subarachnoid hemorrhage (44/F)	2. Mediastinitis after esophageal cancer surgery (55/M)
3. Chronic myeloid leukemia (49/M)	3. Neurodegenerative disorder (44/F)
4. Pulmonary emboli from pelvic veins (48/F)	4. Complications from diabetic ketoacidosis (59/F)
5. Cirrhosis of the liver (54/M)	5. Brain cancer (57/M)
6. Asphyxiation (52/M)	

The age and sex of the patients is in parentheses.

phil infiltration into mechanically dilated regions of rabbit femoral arteries.[5] In support of this hypothesis, patients with heterozygous TM deficiencies have been reported to have an increased risk of early myocardial infarction.[4]

In cell culture, TM and EPCR expression can be down-regulated by inflammatory cytokines exemplified by tumor necrosis factor (TNF)-α.[6] It is known that plasma TNF-α levels are elevated in patients with myocardial infarction.[7] Based on these observations, we postulated that TM and EPCR expression might be reduced in atherosclerotic coronary arteries. To test this hypothesis, we have analyzed endothelial TM and EPCR expression by immunohistochemistry in coronary arteries with and without atherosclerotic lesions.

Materials and Methods

Patients and Samples

A total of six patients with severe coronary artery disease and ischemic cardiomyopathy (ICMP) [mean (SD) age, 53 $\pm$ 7 years; four males and two females] who underwent allograft heart transplantation at the University of Oklahoma Health Sciences Center between 1993 and 1996 were included in the study. The hearts were immersion-fixed and both the left and right coronary arteries were extensively sampled. Three segments each from the left descending coronary artery and the right coronary artery with either stable or vulnerable atherosclerotic plaques were chosen for immunohistochemical studies. Each of these coronary arteries were associated with >50%, but <90%, luminal narrowing. Age matched autopsy cases (n = 6) with no or only insignificant coronary atherosclerosis (no atherosclerotic plaques or occasional plaques with <25% luminal narrowing present) served as the first group of controls. From these control cases, segments of coronary arteries showing no atherosclerotic lesions were chosen for immunohistochemical evaluation. A second control group included five patients with mild overall coronary atherosclerosis, ie, with atherosclerotic coronary lesions causing <50% luminal narrowing. The age and sex distribution, as well as the cause of the death of the patients in the control groups is listed in Table 1. None of the control cases had any clinical or morphological evidence of coronary insufficiency and/or thrombosis. The possibility that some of the underlying conditions leading to death, such as inflammatory disorders or malignancy did alter the endothelial TM and/or EPCR expression in the coronary arteries cannot be excluded. However, many of these disorders are associated with increased cytokine production. Because EPCR and TM can be down-regulated by cytokines, it is likely that the conditions leading to death in the controls would decrease the expression levels for these receptors. All of the control cases were autopsied within 16 hours of death. Sampling and fixation of the coronary arteries from the control cases was similar to that outlined for the hearts of the ICMP cases. From the control cases, segments of coronary arteries showing no atherosclerotic lesions were chosen for immunohistochemical evaluation. Only such samples were selected in which the morphological integrity of the endothelial cells was confirmed by the hematoxylin and eosin-stained sections.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were immunostained for TM, EPCR, and endothelial cell markers (CD31 and CD34) using the streptavidin-biotin-peroxidase method.[8] In brief, 3-μm sections were incubated with 3% hydrogen peroxide to quench endogenous peroxidase activity. The sections were then incubated for 1 hour at room temperature with mouse monoclonal antibodies to detect TM (TM 1009, 7.8 μg/ml), EPCR (1489, 0.2 mg/ml),[9] CD31 (DAKO, Carpinteria, CA), and CD34 (DAKO) expression, respectively. Microwave heat-induced antigen retrieval in citrate buffer, pH 6.0, was required for optimal staining with the anti-CD31 and anti-CD34 antibodies.[10] Primary antibody incubation was followed sequentially by biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 20 minutes, then streptavidin-peroxidase complex (DAKO) for 30 minutes. For negative controls, a monoclonal mouse IgG1 (Bethyl Laboratories Inc., Montgomery, TX) was used at equivalent concentration. Diaminobenzidine was used as chromogen and hematoxylin was used for nuclear counterstain. Each section was assessed by severity of atherosclerosis: severe, >75% luminal narrowing because of atherosclerotic plaque; moderate, 50 to 75% luminal narrowing; and mild, <50% luminal narrowing.

Results

Morphology of the Atherosclerotic Lesions

In the left descending coronary arteries of the ICMP group, luminal narrowing was 75 to 90% in 5 samples and

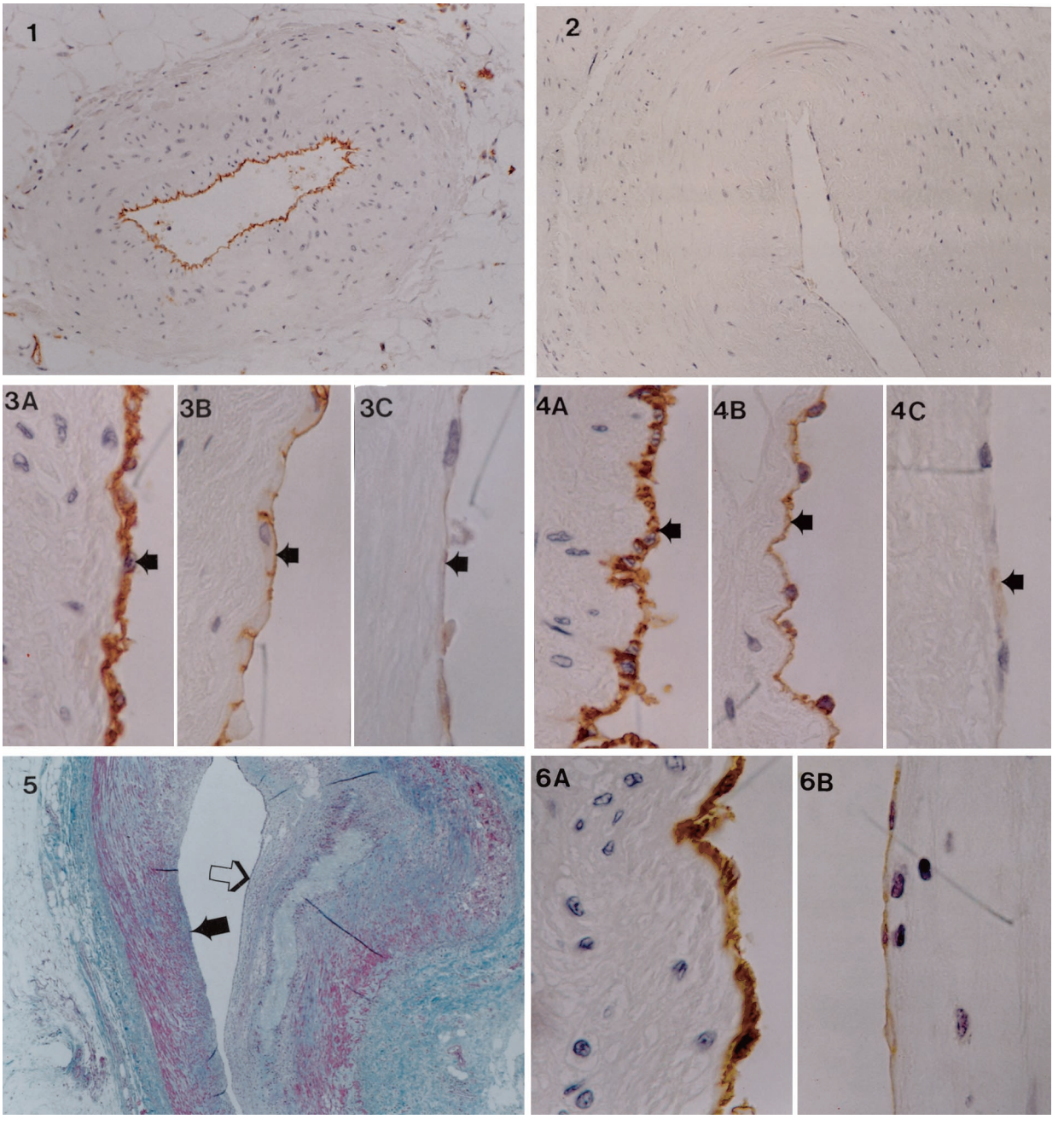

Figure 1. CD31 staining in normal coronary artery. The artery shows strong (3+) endothelial CD31 positivity.

Figure 2. CD31 staining in a coronary artery with severe atherosclerosis. The staining is weak (1+) to absent in the endothelium.

Figure 3. TM staining in normal coronary artery (**A**), and in coronary arteries with moderate (**B**) and severe (**C**) atherosclerosis. The coronary artery with no atherosclerosis (**A**) shows strong (3+) endothelial TM immunoreactivity. The arteries with atherosclerosis reveal moderate (2+) (**B**) and weak (1+) (**C**) endothelial TM positivity. **Arrows** point to the endothelium from the luminal side of the coronary arteries.

Figure 4. EPCR staining in normal coronary artery (**A**), and in coronary arteries with moderate (**B**) and severe (**C**) atherosclerosis. Note that the staining is very similar to that seen for TM. The coronary artery with no atherosclerosis (**A**) shows strong (3+) endothelial EPCR positivity. The arteries with atherosclerosis reveal moderate (2+) (**B**) and weak (1+) to absent (**C**) endothelial EPCR immunoreactivity. **Arrows** point to the endothelium from the luminal side of the coronary arteries.

Figure 5. Coronary artery with a large atherosclerotic plaque affecting primarily the right side (**open arrow**) of the artery (Masson's trichrome). Note that the left side of the artery (**solid arrow**) is relatively unaffected.

Figure 6. EPCR staining in the coronary artery shown in Figure 5. EPCR immunoreactivity is much stronger (3+) in the endothelium overlying the normal part of the vessel (**A**) than in the endothelium from the atherosclerotic portion of the artery (**B**) (1+).

50 to 74% in 13 other samples. In the right coronary arteries of the ICMP group, luminal narrowing was 75 to 90% in 6 samples and 50 to 74% in 12 other samples. None of the plaques was either ulcerated or associated with thrombosis. In five cases, the atherosclerotic plaques had large lipid cores and a thin fibrous capsule.[11] In the control group with mild coronary atherosclerosis but no clinical or morphological evidence of coro-

Table 2. Semiquantitative Analysis of Endothelial EPCR, TM, CD31, and CD34 Expression in Coronary Arteries with no Atherosclerosis (AS), Mild AS, and Clinically Significant (Severe) AS with Ischemic Cardiomyopathy (ICMP)

	EPCR	TM	CD31	CD34
Control group I No AS	2.80 ± 0.40	1.80 ± 0.45	2.69 ± 0.46	2.52 ± 0.50
Control group 2 Mild AS	2.36 ± 0.48	1.59 ± 0.54	2.41 ± 0.60	2.30 ± 0.57
ICMP	1.02 ± 0.31	0.75 ± 0.25	1.05 ± 0.28	1.00 ± 0.33

The intensity of the immunohistochemical stains was graded semiquantitatively by two independent investigators (ZGL and XJZ) in every coronary artery using a scale of 0 to +++ (i.e., 0, 1, 2, and 3). Stains on all specimens were carried out in duplicate. The values in the Table represent average ± SD. Statistical analyses were performed using a Student's *t*-test. $P < 0.05$ was considered statistically significant. The values in the ICMP group are significantly lower for all four markers compared to the corresponding values in the control groups.

nary insufficiency, all of the atherosclerotic plaques were associated with <50% luminal narrowing.

Immunoperoxidase Studies for CD31 and CD34

The endothelial cell staining intensity of both CD31 and CD34 appeared to be lower in the coronary arteries with significant atherosclerosis of ICMP cases than in either of the controls (Figures 1 and 2). Semiquantitative analysis confirmed this with good concordance between the two independent reviewers. In vessels where the sclerotic plaques were not circumferential, the endothelial staining of CD31 and CD34 appeared to be substantially weaker in the atherosclerotic portions of the vessel wall.

Immunoperoxidase Studies for EPCR and TM

In normal control coronary arteries, the endothelial staining for both EPCR and TM was moderate to strong and uniform. Small arterioles serving as internal controls in the normal myocardium showed moderate to strong endothelial staining for both EPCR and TM, similar to that seen in the large coronary arteries. Staining in arteries with severe atherosclerosis was uniformly less intense (Figures 3, 4, 5, and 6) than in those vessels with either mild atherosclerosis or without atherosclerosis. These differences were compared semiquantitatively (Table 2). No significant differences in staining intensity were observed between segments from the left descending coronary arteries *versus* right coronary arteries in the atherosclerotic vessels of the ICMP cases. Furthermore, no significant differences were observed in either EPCR or TM expression in the ICMP cases between arterial segments with more severe *versus* less severe atherosclerosis (ie, arterial segments with 75 to 90% stenosis *versus* segments with 50 to 75% stenosis). In vessels where the sclerotic plaques were not circumferential, the endothelial staining intensity of EPCR and TM was substantially weaker in the atherosclerotic regions of the vessel wall.

Discussion

Most of the acute coronary thrombi with sudden death seem to be attributable to rupture of the fibrous cap overlying a vulnerable plaque.[11] When plaques rupture, the inner core containing tissue factor is thought to initiate the coagulation cascade with subsequent development of local thrombosis. Additional local factors such as reduced endothelial anticoagulant or fibrinolytic activity could shift the thrombogenic balance in favor of increasing thrombosis.

The reduction in endothelial cell TM and EPCR expression demonstrated here provides an example of a shift favoring thrombosis. Although the present study cannot directly demonstrate that reduction in TM and EPCR expression contributes causally to coronary thrombosis, this possibility is supported by a growing number of genetic and animal studies. Monkeys fed an atherogenic diet generate lower levels of activated protein C than control monkeys when both groups are infused with low levels of thrombin.[12] Patients with heterozygous TM deficiency have been reported to have an increased risk of myocardial infarction[13] and specific polymorphisms in the TM gene have been associated with an increased risk of myocardial infarction.[14] Chimeric mice have been produced in which specific vascular regions were made TM-deficient. In these mice, fibrin deposition was observed over the deficient regions.[15] In preliminary clinical studies of EPCR deficiency, patients with EPCR deficiency seemed to have an increased incidence of myocardial infarction.[16] Assuming that the same percent reduction of TM and EPCR levels would occur in the heterozygous patients as occurred in the patients studied here, the heterozygous patients would be nearly devoid of TM or EPCR at the atherosclerotic sites in the vasculature. This situation would approach that seen in the chimeric mice where the TM-deficient endothelium was overlaid with fibrin. The reduction seen in EPCR levels may be particularly favorable to fibrin formation because the rate of protein C activation increases with increasing EPCR concentration even when EPCR is in excess over TM.[17]

From the rabbit experiments in which TM was overexpressed in injured common femoral arteries,[5] it can be inferred that TM plays an important role in preventing leukocyte migration into the vessel wall. Therefore, down-regulation of TM and possibly EPCR would be likely to facilitate the leukocyte influx into the plaques. Activated inflammatory cells have been shown to increase the decay of the plaque cap leading to plaque rupture.[18]

Because of the retrospective nature of the study, we could not address the question of the mechanism of endothelial down-regulation of TM and EPCR. The obser-

vation that other endothelial markers, such as CD31 and CD34, also show decreased expression over atherosclerotic plaques suggests that EPCR and TM may be nonselectively down-regulated in these regions. Active processes may contribute to TM and EPCR down-regulation also. Inflammatory mediators generated locally, such as TNF-α, could down-regulate both TM and EPCR by blocking gene transcription.[6,19] Alternatively, adherence and degranulation of neutrophils can lead to proteolytic release of TM from the endothelium. Endothelial cell stimulation by either thrombin or interleukin-1β (IL-1β) can activate an endothelial cell metalloproteinase that results in EPCR shedding.[20] Because TNF-α can induce IL-1 secretion from endothelium[21] and this in turn can induce EPCR shedding, the elevated TNF-α levels seen in myocardial infarction patients could reduce EPCR expression locally by increasing IL-1. Finally, hypoxia that could result from cardiomyopathy has been shown to decrease TM expression in cell culture.[22] Regardless of the mechanisms involved, the present study indicates that EPCR and TM densities are severely decreased on endothelium overlying atherosclerotic plaques.

Like TM and EPCR, endothelial cell nitric oxide synthase is down-regulated in endothelium overlying the atherosclerotic plaque.[23] Not all endothelial cell proteins, however, are down-regulated at sites of atherosclerosis. Other studies have demonstrated previously that adhesion molecules exemplified by ICAM-1[24] and P selectin[25] are expressed at higher levels on the endothelium overlying atherosclerotic plaque. These changes are likely to work in concert to favor thrombosis and the adhesion of leukocytes and platelets.

Preliminary results from our laboratory indicate that EPCR may have an immunoregulatory function.[26] Soluble EPCR has been shown to interact with leukocytes[27] and blockage of EPCR in a baboon model of sepsis resulted in increased leukocyte infiltration into the tissues.[28] One can hypothesize that decreased endothelial expression of EPCR may contribute to the inflammatory cell influx of the atherosclerotic plaques. By reducing the anticoagulant and anti-inflammatory activity of the endothelium overlying the atherosclerotic plaque, the down-regulation of EPCR and TM could facilitate both plaque rupture and increase the size of the resultant thrombus.

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