Expression of endothelial protein C receptor and thrombomodulin in human coronary atherosclerotic plaques

Piera Angelica Merlini, Marco L. Rossi*, Elena M. Faioni**, Franca Franchi***, Ezio Bramucci§, Stefano Lucreziotti§, Eugenia Biguzzi***, Pier Mannuccio Mannucci***, Diego Ardissino§§

*Division of Cardiology, Niguarda Ca' Granda Hospital, Milan, *Division of Cardiology, Istituto Clinico Humanitas, Milan, **Division of Internal Medicine, San Paolo Hospital, Milan, ***Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, IRCCS Ospedale Maggiore and Department of Internal Medicine, University of Milan, §Division of Cardiology, IRCCS Policlinico San Matteo, Pavia, §§Division of Cardiology, University of Parma, Parma, Italy*

Key words:
Atherosclerosis; Genes; Thrombosis.

Background. **The expression of selected genes in human coronary atherosclerotic plaques may help to clarify the evolution of atherogenesis and the causes of thrombogenesis on some fissured plaques. The aim of this study was to analyze the expression of the genes known to participate in inflammation and hemostasis: thrombomodulin and endothelial protein C receptor, E- and P-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), tissue factor and plasminogen activator inhibitor-1 (PAI-1).**

Methods. **RNA was extracted and reverse-transcribed from 27 atherectomized human coronary atherosclerotic plaques. The genes were specifically amplified together with a housekeeping gene.**

Results. **Thrombomodulin was not expressed in the 8/27 plaques from which RNA could be obtained. The levels of expression of tissue factor, ICAM-1, P- and E-selectin, and PAI-1 were low, whereas those of endothelial protein C receptor and VCAM-1 were high.**

Conclusions. **RNA may be extracted from *ex vivo* atherosclerotic plaques. In addition to anticoagulation, endothelial protein C receptor may play an important inflammation-related role in plaque development.**

(Ital Heart J 2004; 5 (1): 42-47)

Received September 16, 2003; revision received December 1, 2003; accepted December 9, 2003.

Address:

Dr. Marco L. Rossi

U.O. di Emodinamica
Istituto Clinico Humanitas
Via Manzoni, 56
20089 Rozzano (MI)
E-mail: marcolrossi@hotmail.com

The clinical manifestations of coronary atherosclerosis range from stable to unstable angina, myocardial infarction, and sudden cardiac death. Stable angina is related to the gradual encroachment of the vessel lumen by progressively growing atherosclerotic plaque, whereas the pathological and angiographic correlates of unstable angina and myocardial infarction are atherosclerotic plaque fissures and thrombosis[1-9].

It is not clear what causes plaques to change from a stable to an unstable state, but inflammation and thrombosis may both be involved. It is likely that a combination of acquired and inherited systemic causes are responsible for creating an individual predisposition to plaque rupture and subsequent thrombosis, but plaque composition and the local loss of the anticoagulant properties of normally functioning endothelium may play an important role in precipitating the thrombotic response to rupture. The best approach to improving our understanding of the role of local factors would be to study the local expression of the genes involved in thrombosis.

Studies evaluating the expression of selected plaque genes have so far been hindered by the limited *ex vivo* availability of atherosclerotic plaques. Immunohistochemistry, *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) may all contribute to the identification of the genes and gene products present in different plaques, but such approaches have often been restricted to studying the expression of only one or at most a few genes at a time. Furthermore, most of the studies have been performed using animal models, carotid-derived plaques or *post-mortem* samples.

We here describe a relatively quantitative RT-PCR approach to the study of gene expression in atherectomized human coronary artery atherosclerotic plaques obtained from patients with stable or unstable ischemic heart disease. The aim of the study was to evaluate the feasibility of a

multiple quantitative approach to gene expression in human coronary plaques, particularly the expression of the genes participating in hemostasis and inflammation at the endothelial level.

Methods

Study population. The study population was drawn from a cohort of consecutive patients undergoing directional coronary atherectomy between September 1998 and September 1999 at the Division of Cardiology, IRCCS Policlinico San Matteo, Pavia, Italy. In order to maximize the differences between the two extremes of the clinical spectrum of ischemic heart disease, we only included patients with stable or unstable disease defined on the basis of clinical, morphological and angiographic criteria.

Stable disease was defined as the presence of stable angina and stable plaque at angiography. Stable angina was defined as a history of chest pain induced by exercise or usual daily activity lasting > 6 months with the development of at least a 1 mm ST-segment depression during the exercise test and with significant coronary artery disease at angiography. Stable plaque was diagnosed at angiography in the presence of plaques with smooth borders.

Unstable disease was defined as the presence of unstable angina and an angiographically unstable plaque morphology. Unstable angina was defined as chest pain occurring at rest, accompanied by transient ischemic ECG changes (ST-segment elevation or depression > 1 mm 0.08 s after the J point or the pseudonormalization of previously negative T waves)[10] and abnormal troponin T values. An unstable plaque morphology was diagnosed at angiography in the case of irregular borders, overhanging edges, ulcerations or thrombosis[8].

Coronary angiography and directional coronary atherectomy. Selective coronary arteriography was performed in multiple views using the Judkins technique. A more than 70% diameter narrowing was considered significant coronary artery stenosis. The angiographic morphology of the lesions was prospectively assessed. Directional coronary atherectomy was performed using standard clinical procedures. Briefly, after the insertion of a 300 cm long guidewire, the atherectomy device was positioned at the level of the stenosis and the support balloon was inflated up to 1 atm. The cutter was then retracted and the inflation pressure of the balloon increased to a maximum of 3 atm. The driving motor was activated and the rotating cutter slowly advanced in order to resect and collect the protruding plaque in the collection chamber located at the tip of the catheter. After each pass, the balloon was deflated and either removed or repositioned. The extracted plaques were treated with RNase-free material and immediately frozen in liquid nitrogen.

Plaque processing and RNA extraction. None of the laboratory staff were aware of the clinical and angiographic diagnoses until the study was completed. After atherectomy, the plaques were flushed in RNAzol™ and immediately frozen in liquid nitrogen until use. The plaques were disrupted on ice in 0.5 ml using a Dounce homogenizer, and RNA was extracted by standard methods (RNAzol™ B Isolation of RNA kit, TEL-TEST, Inc., Friendswood, TX, USA) with the addition of glycogen (20 µg/sample) during the precipitation step. After extraction, each RNA sample was re-suspended in water and RNase inhibitor (40 U/sample, Roche Molecular Systems, Monza-MI, Italy), and stored at -80°C.

RNA quality and yield were evaluated by means of agarose gel electrophoresis and SYBR Gold staining (10^{-4} in MOPS). The RNA was visualized using a FluorImager (Molecular Dynamics Inc., Milan, Italy), and quantified by means of Image Quant (Molecular Dynamics Inc.) against rat small intestine RNA standards ranging from 1200 to 18.75 ng. Three independent operators evaluated the quality of the RNA by visually comparing the amount of RNA on gels at 28 and 18 S with that visible at a lower molecular weight (which denotes degradation). The quality of each sample was independently defined as "good", "intermediate" or "poor". Only the plaques whose RNA was classified as being of good or intermediate quality were included in the study.

Reverse transcription and amplifcation. RNA was reverse transcribed using the SMART™ PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA) and oligo-dT primers. DNA fragments were amplified from the following genes: endothelial protein C receptor (EPCR), thrombomodulin (TM), tissue factor (TF), plasminogen activator inhibitor-1 (PAI-1), E-selectin (E-sel), P-selectin (P-sel), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). The housekeeping glyceraldehyde-6-phosphate dehydrogenase (GAPDH) gene was co-amplified with each candidate gene in a single PCR tube. The conditions were such that the candidate and housekeeping gene in each co-amplification were amplified with the same efficiency, and the amount of amplification product for each PCR reaction was proportional to the amount of specific target cDNA[11]. The target cDNA in each PCR reaction was considered as being a maximum of 3 ng, corresponding to 1 µl of a 1:60 dilution of the total cDNA obtained from each plaque. The following (forward and reverse) primers were used:

- EPCR: 5'ATGTCTTCTTCGAAGTGGCTG3' and 5'GAGTGCGGTTGTAGGCATTG3'
- TM: 5'CAGGTGCCAGATGTTTTGCA3' and 5'ACGGCCGGAGGAGTCAAGGT3'
- TF: 5'CACTAAGTCAGGAGATTGGAAAAG3' and 5'TCTCCAGGTAAGGTGTGAACTC3'
- PAI-1: 5'CCTCCGGCATCTGTACAAG3' and 5'TCTCCACCTTCTGAAAAGTCC3'

• E-sel: 5'GCTTCAAGAGACAGAGTTTCTG3' and 5'AGCTGAACACTACTTCGGCTG3'
• P-sel: 5'GGCAGCATGGACTTATCATTAC3' and 5'ATCCAGTAGTAGGAGCTGTAG3'
• ICAM-1: 5'CGGTATGAGATTGTCATCATCAC3' and 5'CTTGTGTGTTCGGTTTCATGG3'
• VCAM-1: 5'CTCCTGAGCTTCTCGTGCT3' and 5'CATAGATGGGCATTTCTTTCC3'
• GAPDH: 5'CTGCTGTAGGCTCATTTGCAG3' and 5'ACTTCTCATGGTTCACACCC3'

Polymerase chain reaction conditions. Each 30 ml of PCR mixture contained 2.5 mmol/l $MgCl_2$, 200 μmol/l dNTP, 0.1 mg/ml bovine serum albumin, 1.5 U TaqGold (Applied Biosystems Italia SpA, Monza-MI, Italy), and approximately 3 ng of target cDNA. The primer concentrations were: TM or EPCR 2.5 pmol and GAPDH 7.0 pmol; TF 18.0 pmol and GAPDH 12.5 pmol; PAI-1 25 pmol and GAPDH 4.7 pmol; E-sel 2.7 pmol and GAPDH 6.0 pmol; P-sel 2.7 pmol and GAPDH 10.0 pmol; VCAM-1 2.5 pmol and GAPDH 7.0 pmol; ICAM-1 2.5 pmol and GAPDH 8.0 pmol. Touch-down PCR was performed by means of initial denaturing at 95°C for 9 min. The subsequent cycles were denaturing: 95°C for 30 s; annealing: two cycles at 66°C for 30 s, two cycles at 64°C for 30 s, two cycles at 62°C for 30 s, two cycles at 60°C for 30 s, and 26 cycles at 58°C for 30 s; extension: 72°C for 40 s, with a final extension of 7 min at 72°C. The amplified bands were electrophoretically analyzed on 2.5% agarose gels and visualized by means of ethidium bromide staining. Each band was quantified by scanning (Image Master VDS, Pharmacia Biotech, Milan, Italy), and its integrated optical density was measured. The results for each gene were expressed as percentages of the GAPDH integrated optical density amplified in parallel. The optical density readings were stopped before reaching saturation.

Positive amplification controls. A positive control was amplified in parallel in each PCR using cDNA from the following tissues: placenta for TF, TM and EPCR; peripheral blood mononuclear cells for ICAM-1; resting endothelial cells for PAI-1; and 10 ng/ml of tumor necrosis factor-stimulated endothelial cells (harvested at 24 hours) for E-sel, P-sel and VCAM-1.

Genomic contamination controls. Contamination by genomic DNA was checked in the genetic material derived from each plaque by means of amplification using the following primers designed within an intron:
• 5'CTCATCCTCAGCTCCTAATGCT3'
• 5'AGGACTCAGACCCCTGCCAGACAC3'

Results

Patients. Twelve patients were prospectively classified as having stable disease: 11 men and 1 woman with a median age of 55 years (range 40-69 years). The risk factors for coronary disease were hypertension in 5 cases, smoking in 6, dyslipidemia in 6, a positive family history in 6, and diabetes in 2. Four of the 12 patients had had a previous myocardial infarction. All were receiving chronic statin treatment, and 2 were receiving ACE-inhibitors.

Fifteen patients were prospectively classified as having unstable disease: 13 men and 2 women with a median age of 57 years (range 43-76 years). The risk factors for coronary disease were hypertension in 6 cases, smoking in 9, dyslipidemia in 6, a positive family history in 7, and diabetes in 5. Nine of the 15 patients had had a previous myocardial infarction. All of the atherectomized lesions had an angiographically "complex" morphology, and 12 showed visual signs of thrombosis. None of the unstable angina patients had been on chronic treatment with statins or ACE-inhibitors.

RNA was extracted from the 27 plaques and evaluated in terms of quality and total amount as described in the Methods section. The eight plaques that produced measurable yields of RNA of good or intermediate quality were chosen for the subsequent study of gene expression. The characteristics of the 8 patients from whom the plaques were taken are shown in table I.

Table I. Clinical and demographic characteristics of the patients whose plaques were used in the study. A classification of the RNA extracted from each plaque is also included.

No. patient	Patient group	Age (years)	Sex	Previous MI	Risk factors for MI (no.)	RNA quality
24	Unstable	70	M	Yes	Yes (1)	Good
45	Unstable	52	M	Yes	Yes (1)	Intermediate
54	Unstable	55	F	No	Yes (3)	Intermediate
55	Unstable	76	M	Yes	No	Intermediate
32	Stable	53	M	Yes	Yes (2)	Good
35	Stable	43	F	No	No	Intermediate
43	Stable	69	M	No	Yes (1)	Intermediate
51	Stable	48	M	No	Yes (2)	Intermediate

MI = myocardial infarction.

Polymerase chain reaction conditions. For each amplification reaction aimed at co-amplifying the candidate and GAPDH gene, the conditions were such that the amplification efficiency of both was equivalent, and the PCR was in the exponential part of the product-to-cycle number curve (a linear relationship between target and product). To verify the equivalence of amplification efficiency, several PCRs were performed for each pair (the candidate and housekeeping gene) in order to establish the correct relative concentrations of the PCR components. The amount of target cDNA was set at 3 ng, and the PCR products were run on agarose gels, quantified by scanning and compared. The PCR products were quantified at different cycles for each PCR reaction: typical examples of TF and GAPDH are shown in figures 1 and 2. The amount of PCR product (equivalent for both genes) increases until reaching a plateau, and the conditions were set for the amplification of plaque cDNA after 34 cycles, which is well within the exponential part of the curve.

Quantitative reverse transcriptase-polymerase chain reaction. Table II shows results of the parallel specific amplifications of the candidate genes and GAPDH from each of the eight plaques. EPCR and VCAM-1 had the highest values, and TF and TM the lowest. All the positive and negative controls behaved appropriately. As expected, genomic DNA in the control experiment could not be amplified (not shown).

Discussion

Two conclusions may be drawn from this study. First of all, quantitative RT-PCR is feasible in at least one third of the plaques atherectomized from human coronary arteries and makes it possible to compare the expression of the multiple genes potentially involved in the thrombogenesis of coronary atherosclerotic plaques. This is important because obtaining *ex vivo* material from human coronary atherosclerotic plaques allows the validation of previous animal or *post-mortem* results, with all of their limitations. Very few studies have used RT-PCR to analyze gene expression in coronary plaques, and all of them have dealt with a maximum of two genes and have provided no quantitative data.

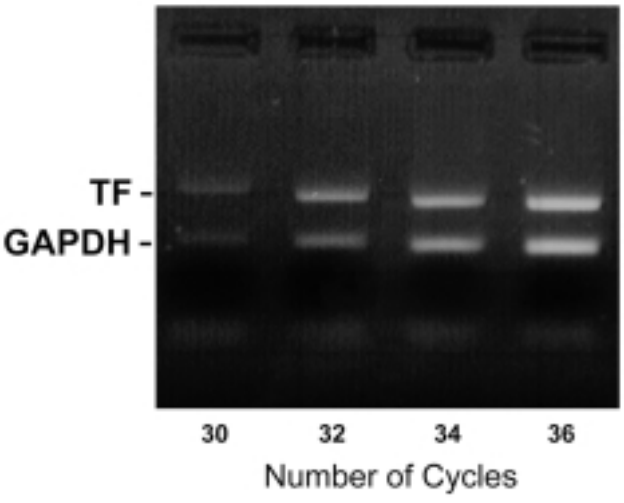

Figure 1. *Co-amplification of tissue factor (TF) and glyceraldehyde-6-phosphate dehydrogenase (GAPDH) at different polymerase chain reaction cycles showing the similar efficiency of amplification for the two genes under the reverse transcriptase-polymerase chain reaction conditions described in the Methods section. The amplified fragments obtained at different cycles were run on 2.5% agarose gels, stained with ethidium bromide and visualized by means of a UV scanner.*

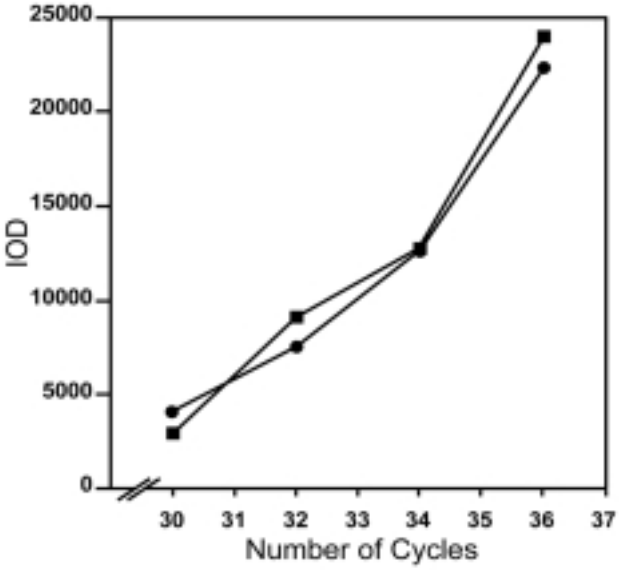

Figure 2. *Quantitation in the same experiment as that shown in figure 1. The integrated optical density (IOD) results were plotted against the number of polymerase chain reaction cycles.*

One of the limitations of our technique is that two thirds of the samples could not be included in the analysis, and even the RNA that was examined had some degree of degradation. It is likely that the high frequency of degradation was related to pre-RNA extraction variables, such as the time of handling during the atherectomy procedure, the time from sampling to freezing, and the time spent in liquid nitrogen. As some of these variables can be checked and modified, it may be possible to improve RNA quality and yields in the future.

The second conclusion specifically concerns the gene expression results. It was not possible to compare the plaques taken from patients with stable or unstable ischemic disease because of the small number of samples. It has been previously shown that atherosclerotic plaques contain RNA of TF and E-sel as evaluated by means of *in situ* hybridization, and the corresponding proteins as evaluated by means of immunohistochemistry or direct antigen/function measurements[12-21]. TF has been studied in depth because of its central role in coagulation, and various studies have reported its presence in atherosclerotic plaques, in close association

Table II. Quantitative analysis of gene expression from coronary atherosclerotic plaques.

No. plaque	Plaque characteristics	EPCR	TM	TF	PAI-1	E-sel	P-sel	VCAM-1	ICAM-1
24	Unstable	215	0	3	41	0	14	88	14
45	Unstable	1254	7	3	12	0	25	145	22
54	Unstable	100	0	4	15	0	0	256	4
55	Unstable	280	0	3	18	0	5	88	12
32	Stable	224	0	0	20	0	13	270	7
35	Stable	219	4	1	8	0	3	285	20
43	Stable	49	0	0	12	0	5	167	7
51	Stable	140	0	0	17	0	0	480	32

Data are expressed in percent expression of glyceraldehyde-6-phosphate dehydrogenase amplified in parallel measured as integrated optical density. Plaques were defined as stable or unstable on the basis of the clinical characteristics of the patients they were taken from and of the angiographic morphology. EPCR = endothelial protein C receptor; E-sel = E-selectin; ICAM-1 = intercellular adhesion molecule-1; PAI-1 = plasminogen activator inhibitor-1; P-sel = P-selectin; TF = tissue factor; TM = thrombomodulin; VCAM-1 = vascular cell adhesion molecule-1.

with the lipid-rich core or macrophage infiltrate[12-14]. However, its frequency and amount vary, possibly depending on the population of sampled patients and the experimental method used[22-24]. We found TF RNA in all of our unstable plaques, although its level of expression was low in comparison with that of the GAPDH housekeeping gene. The levels of expression of PAI-1, P-sel and ICAM-1 were also low, whereas those of VCAM-1 and EPCR were higher.

The role of PAI-1 in plaques is not clear[25-28], but it has been postulated that it may contribute to the maintenance of the prothrombotic phenotype of the plaque and participate in regulating extracellular matrix proteolysis. Leukocyte adhesion molecule expression is an essential step in the pathogenesis and evolution of atherosclerotic plaque and is mediated by cytokines which have been shown to be abundant in plaque (interleukin-1, tumor necrosis factor-α)[29]. P-sel is involved in leukocyte adhesion and is present on platelets and endothelial cells; ICAM-1 is expressed on the endothelium and promotes the adhesion of monocytes, lymphocytes and neutrophils; besides, VCAM-1 binds monocytes and T-lymphocytes[30]. In all eight of our plaques we detected higher levels of VCAM-1 expression in comparison with the other cell adhesion molecules, which suggests a selective recruitment of inflammatory cells in atherosclerosis and the possibly different roles of the various adhesion molecules.

The two endothelial receptors involved in anticoagulant pathways (EPCR and TM) showed the same differential expression of interrelated genes. EPCR[30] is an endothelial cell receptor localized in arterial and venous endothelia where it participates in protein C activation by binding protein C and increasing the catalytic efficiency of the thrombin-TM complex. It is likely that it plays a central role in the anticoagulant protein C pathway. Like TM, it is downregulated by the cytokine tumor necrosis factor[31-33], and so it is surprising that (unlike TM, which could not be amplified in our experimental setting) it was highly expressed in the plaques of our patients regardless of whether they had stable or unstable angina.

It is tempting to speculate that the two receptors play different roles in the history of plaque evolution. TM is downregulated by interleukin-1 and/or tumor necrosis factor during the inflammatory processes that are a key feature of plaque formation, thus shutting down the anticoagulant effect of the endothelium on the plaque. On the other hand, in addition to participating in protein C activation, EPCR may have still-undefined functions possibly related to inflammation. The observation that the sequence and structure of EPCR place it in the CD1/major histocompatibility complex superfamily[32] is consistent with this. A recent study showing that a mutation in the EPCR gene is associated with an increased risk of myocardial infarction in the young[33] indicates its important role in ischemic heart disease.

In conclusion, RT-PCR of several genes from *ex vivo* human coronary atherosclerotic plaques is feasible and may provide useful information regarding the plaque characteristics in individual patients. The anticoagulant EPCR gene is expressed in the coronary atherosclerotic lesions of patients with ischemic heart disease at similar levels to the proinflammatory VCAM-1 gene. This suggests that, in addition to its anticoagulant properties, EPCR may play a role in the inflammation/repairing process. The localization of the expression of these genes by means of *in situ* hybridization and immunohistochemistry will provide further information concerning their possible role in atherogenesis.

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