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High Telomerase Activity in Neutrophils From Unstable Coronary Plaques

Maria Lucia Narducci, MD,* Annalisa Grasselli, PHD,*¶ Luigi Marzio Biasucci, MD, FACC,* Antonella Farsetti, MD,‖¶ Antonino Mulè, MD,† Giovanna Liuzzo, MD,* Giuseppe La Torre, MD,§ Giampaolo Niccoli, MD,* Rocco Mongiardo, MD,* Alfredo Pontecorvi, MD,‡ Filippo Crea, MD, FACC*

Rome, Italy

Objectives We evaluated telomerase activity in circulating polymorphonuclear neutrophils (PMN) and in PMN isolated from coronary atherosclerotic plaques by a novel approach.

Background Delayed apoptosis of PMN have been demonstrated in unstable angina (UA). These cells have a finite lifespan with low telomerase activity, a polymerase that extends telomeres, structures essential for cell aging. Reactivation of telomerase has been associated with resistance to apoptosis.

Methods We studied 20 patients with UA and 6 patients with chronic stable angina (SA), undergoing a percutaneous coronary intervention. Circulating PMN were isolated from venous blood and PMN derived from coronary plaque were isolated from washing medium of angioplasty balloons.

Results Telomerase activity was higher in coronary plaque PMN of UA patients than in coronary plaque PMN of SA patients (122.7, range 20.5 to 3,696; and 47.7, range 16 to 212.6, respectively, $p = 0.001$) and higher than in peripheral PMN of SA patients (122.7, range 20.5 to 3,696 vs. 59, range 16.5 to 132.5, $p = 0.001$). We found a statistically significant difference between venous and coronary plaque PMN telomerase activity in UA patients ($z = -2.875$; $p = 0.004$). Among UA patients, a shorter time interval from symptom onset to coronary PMN sampling was the only independent predictor of high telomerase activity in coronary plaque PMN ($p < 0.001$, $R^2 = 0.75$).

Conclusions In UA patients, telomerase activity is high in coronary plaque PMN, while it is low in peripheral PMN. Telomerase reactivation in resident PMN resulting in a prolonged lifespan might play a key role in the early phases of instability. (J Am Coll Cardiol 2007;50:2369–74) © 2007 by the American College of Cardiology Foundation

Telomeres are specialized deoxyribonucleic acid (DNA)-protein structures that contain noncoding TTAGGG repeats and associated proteins, essential for chromosome stability. Depending upon the cellular context, telomere shortening may lead to cell senescence or apoptosis. Telomere maintenance is primarily achieved by telomerase, a ribonucleoprotein with reverse transcriptase activity that uses its internal ribonucleic acid component as a template for the synthesis of telomeric DNA. Telomerase activity is present during early development and in adult germline and stem cells of self-renewing tissues, but absent or functionally insufficient in adult somatic cells. In the absence of the enzyme, telomeres shorten with cell division, a process that may act as a mitotic clock and signal entry into senescence. Shortening of telomeres is thus held responsible for the limited lifespan of somatic cells in culture and has also been associated with organismal aging (1–3). The mechanisms whereby telomerase controls these processes are beginning to be understood and include effects on the signaling cascades that regulate apoptosis. Indeed, inhibition of telomerase and the ensuing telomere shortening below a critical length results in apoptosis in various cell types, whereas induction of telomerase activity is associated with resistance to apoptosis (4,5).

In particular, polymorphonuclear neutrophils (PMN) have a finite lifespan and typically die by undergoing apoptosis. Thus, PMN apoptosis represents a control mechanism limiting the toxic potential of these short-lived, terminally differentiated cells (6,7). Post-mortem studies have recently shown PMN infiltration in unstable but not in stable atherosclerotic plaques, suggesting their possible

From the *Institute of Cardiology, †Division of Anatomic Pathology and Histology, ‡Institute of Medical Pathology, and the §Institute of Hygiene, Catholic University of Sacred Heart, Rome, Italy; ‖Neurobiology and Molecular Medicine Institute, National Council of Research, Rome, Italy; and the ¶Department of Experimental Oncology, Regina Elena Cancer Institute, Rome, Italy. This work was partially supported by "Associazione Italiana Ricerca sul Cancro."

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Abbreviations and Acronyms

DNA = deoxyribonucleic acid
PCI = percutaneous coronary intervention
PMN = polymorphonuclear neutrophils
SA = stable angina
TRAP = telomeric repeat amplification protocol
UA = unstable angina

role in destabilization of coronary plaque (8). Accordingly, several clinical studies have consistently demonstrated that acute coronary syndromes are associated with systemic evidence of PMN activation (9–12). More recently, Garlichs et al. (13) observed systemic activation and delayed apoptosis of PMN in patients with unstable angina (UA) as compared with patients with stable angina (SA).

In order to characterize the mechanism of delayed PMN apoptosis in acute coronary syndromes, we assayed telomerase activity in PMN isolated from peripheral blood in patients with chronic SA and in patients with UA. In the same patients, we also evaluated telomerase activity in PMN isolated directly from coronary atherosclerotic plaques obtained during percutaneous coronary intervention (PCI), using a novel technique.

Methods

Study population. We enrolled 26 consecutive patients undergoing PCI: 20 patients with Braunwald class IIB or IIIB UA and 6 patients with SA for at least 6 months before admission. We excluded all subjects with chronic or acute infections or an inflammatory condition, as defined elsewhere (14). Patient characteristics are reported in Table 1 (15).

The protocol was approved by the ethics committee of the Catholic University of Rome, and all patients gave written informed consent.

Peripheral cell isolation. During coronary angiography, all patients underwent blood sampling from the right femoral artery and vein. Peripheral blood PMN were isolated by a single-step density gradient procedure using Polymorphprep separation medium (Nycomed Pharma AS, Oslo, Norway). After centrifugation at 500 × *g* for 30 min at 20°C, the mononuclear cell band and PMN were retrieved and washed twice in phosphate-buffered saline. Contaminating erythrocytes were removed by hypotonic lysis. Peripheral PMN pellets were stored at −80°C.

Coronary plaque cell isolation and PMN identification. After coronary angiography, all unstable patients underwent PCI on the culprit stenosis and stable patients on at least 1 critical stenosis. A standard dose of heparin (5,000 IU) was used in all patients. Stent deployment was preceded by predilation, and angioplasty balloons used for predilation were similar in the 2 groups of patients (Maverick, Boston Scientific, Natick, Massachusetts). Our novel protocol for PMN collection was characterized by the following steps:

1. Inflation of pre-dilation balloon for 20 s at a mean of 10 (6 to 14) atmospheres

Table 1 Demographic and Clinical Characteristics of Patients

	Unstable Angina (n = 20)	Stable Angina (n = 6)	p Value
Age (yrs)	67 ± 9	67 ± 8	NS
Gender (M/F)	3/20	2/6	NS
Risk factors , n (%)			
Family history of IHD	5 (25%)	1 (16%)	NS
Hypercholesterolemia	13 (65%)	5 (83%)	NS
Diabetes	8 (40%)	2 (33%)	NS
Hypertension	16 (80%)	3 (50%)	NS
Obesity (BMI >25 kg/m^2)	3 (15%)	1 (16%)	NS
Smoking (current status)	0 (0%)	0 (0%)	NS
Medication, n (%)			
Beta-blockers	18 (90%)	5 (83%)	NS
Calcium blockers	6 (30%)	3 (50%)	NS
ACE inhibitors	13 (65%)	4 (66%)	NS
Aspirin	17 (85%)	4 (66%)	NS
Other antiplatelet agents	16 (80%)	3 (50%)	NS
Lipid-lowering therapy	18 (90%)	6 (100%)	NS
Hormonal therapy*	1 (5%)	2 (33%)	NS
Coronary angiographic lesions†, n (%)			
Type A	5 (25%)	3 (50%)	NS
Type B	13 (65%)	3 (50%)	NS
Type C	2 (10%)	0 (0%)	NS

*Hormonal therapy is represented by finasteride, a type 2 5alpha-reductase inhibitor; †Coronary angiographic lesions are classified according to morphology in type A, B, and C (15).
ACE = angiotensin-converting enzyme; BMI = body mass index; IHD = ischemic heart disease.

2. Deflation of pre-dilation balloon that was immediately pulled back inside the guiding catheter and collected in appropriate tubes with 5 ml of Cytolyt solution (Cytyc Corp., Boxborough, Massachusetts)

The persistency time of balloon inside the guiding catheter was extremely short, <5 s, thus minimizing the possibility of contamination by blood constituents.

In order to confirm the plaque source of PMN, we tested in a subgroup of patients the presence of these inflammatory cells after inflating the balloon inside the guiding catheter, placed in the ascending aorta for the same duration and inflation pressure. We failed to find PMN from this washing medium isolated by the same technique.

After centrifugation of washing medium at 1,200 × *g* for 10 min, the cell pellet was added to 20 cc of PreservCyt solution (Cytyc Corp.) in a plastic tube mounted on a polarized microscope slide. Several sedimentation intervals were tested, and the optimal PMN isolation was obtained with 4-h decantation. The procedure based on different cell sedimentation velocity yielded a minimum of 50 up to 200 PMN (mean 125) per slide, corresponding to >90% of the total cellularity. Immunocytochemistry with myeloperoxidase MPO-7 (Dako Laboratories, Glostrup, Denmark) antibodies was performed to confirm PMN morphological features. The immunocytological staining was performed using a standard streptavidin-biotin-peroxidase method (DAKO, Copenhagen, Denmark) (Fig. 1).

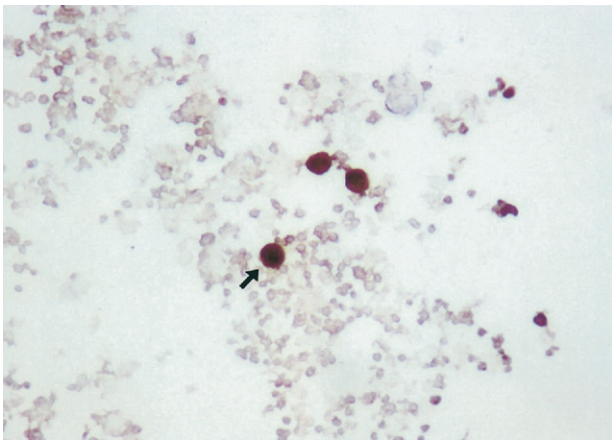

Figure 1 **Immunostaining of Coronary Plaque PMN With Myeloperoxidase Antibodies**

Immunostaining for myeloperoxidase MPO-7 antibodies **(purple)** reveals staining of all cells isolated by direct washing of angioplasty balloon used for coronary revascularization, indicating that MPO-7–positive cells are neutrophils **(arrow)**. Original magnification: ×444. PMN = polymorphonuclear neutrophils.

As a control we used washing medium of the distal portion of dilatation catheter without balloon in 6 cases; in these cases we did not obtain PMN but red blood cells from the washing medium.

Telomeric repeat amplification protocol (TRAP). Telomerase activity was analyzed using the TRAP described by Kim et al. (16). Briefly, extracts from peripheral or coronary PMN were prepared by detergent lysis. After incubation on ice, the lysate was centrifuged, and the supernatant was immediately used to evaluate telomerase activity in this assay. The reaction was carried out using 2 μg of protein extractions in 50 μl of reaction mixture, to which an internal telomerase assay standard was added for estimation of telomerase activity and identification of any false-negative samples containing Taq polymerase inhibitors.

Similar results were obtained using different concentrations (0.2, 0.5, 1, 2, 5, and 10 μg) of protein extracts (data not shown). Quantitative analysis was performed with the ImageJ 1.24 software (National Institutes of Health, Bethesda, Maryland), and telomerase activity was quantified by measuring the signals of telomerase ladder bands and expressed as densitometric values. The relative telomerase activity was calculated as the ratio to the internal standard. As positive and negative controls, 0.1 μg of protein from telomerase-positive HeLa cells was assayed before and after heat inactivation. All assays were performed by a single investigator, who was blinded to patients' characteristics.

Statistical analysis. Chi-square test was used for comparing the frequency distribution of categorical variables among groups. Telomerase activity values were not normal distributed, and the data are reported as medians and ranges.

Comparisons of variables not showing normal distribution between UA and SA patients were performed with Kruskal-Wallis test; in the case of overall between-groups significant differences at Kruskal-Wallis test, direct comparisons were performed by Mann-Whitney test, with Bonferroni correction.

Within-subjects comparisons of variables not showing normal distribution in the 2 populations were performed with paired nonparametric Wilcoxon signed rank test.

Univariate correlation between telomerase activity and other variables was performed using nonparametric Spearman rank test.

Multivariate analysis was performed using linear regression after log transformation of the dependent variable, telomerase activity, since the distribution was skewed. We used a stepwise procedure (backward elimination) including in the model age and gender (as potential confounders or effect modifiers) and parameters that at univariate analysis were related to telomerase activity ($p < 0.10$). The goodness of fit of the model was assessed using the R^2 statistic and the distribution of the unstandardized residuals. The significance level for p was set at $p < 0.05$. The statistical analysis was performed using SPSS software, release 12.0 (SPSS Inc., Chicago, Illinois).

Results

Telomerase activity, measured by TRAP assay, in PMN isolated from venous, arterial blood or by direct washing of angioplasty balloons was represented in Figure 2. Extracts from PMN isolated by direct washing of angioplasty balloons showed high telomerase activity in UA patients. Telomerase activity was barely detectable in PMN derived from angioplasty balloons of patients with SA and in PMN derived from venous and arterial blood of patients with UA and SA (Fig. 2).

Telomerase activity was higher in coronary plaque PMN of patients with UA than in coronary plaque PMN of patients with SA (122.7, range 20.5 to 3,696; and 47.7, range 16 to 212.6, respectively, $p = 0.001$) and higher than in peripheral PMN of SA patients (122.7, range 20.5 to 3,696 vs. 59, range 16.5 to 132.5, $p = 0.001$). Particularly, we found a statistically significant difference between venous and coronary plaque PMN telomerase activity in UA patients ($z = -2.875$, $p = 0.004$) (Fig. 3).

Telomerase activity in arterial PMN was similar to that observed in venous PMN (data not shown).

Among UA patients, a significant inverse correlation ($p < 0.001$) between telomerase activity in coronary plaque PMN and time interval from last episode of angina and PMN sampling by direct washing of angioplasty balloons was found at univariate analysis. In contrast, no correlation was found between cardiovascular risk factors, medical therapy, angiographic type of coronary lesions, and telomerase activity (Table 2). At multiple linear regression analysis, the following variables were included in the model: time interval from last episode of angina and coronary plaque PMN sampling, diabetes, age, and gender; time

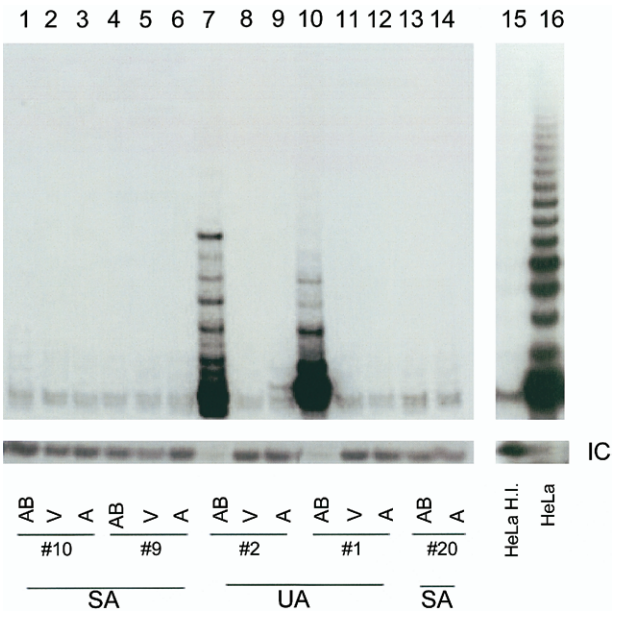

Figure 2 **High Telomerase Activity in Coronary Plaque PMN of Patients With UA**

Cell extracts derived from polymorphonuclear neutrophils (PMN) isolated from arterial (A) or venous blood (V) or directly from angioplasty balloons (AB) of unstable angina (UA) and chronic stable angina (SA) patients were assayed for telomerase activity by telomeric repeat amplification protocol assay in the presence of internal control (IC) (36 base pairs). As positive and negative controls, cell extracts (0.1 μg) from telomerase-positive HeLa cells were assayed before and after heat inactivation (HeLa and HeLa H.I.), respectively. Panels relative to the IC are shown at a lower detection exposure. Telomerase activity was barely detectable in PMN of patients with chronic SA (lanes 1 to 6, 13, and 14) and in arterial and venous PMN in patients with UA (lanes 8 and 9 and 11 and 12). In contrast, telomerase activity was high in PMN from the coronary atherosclerotic plaques in patients with UA (lanes 7 and 10).

interval from the last episode of angina and coronary plaque PMN sampling remained the only independent predictor of telomerase activity in coronary plaque PMN ($p < 0.001$, $R^2 = 0.75$) (Fig. 4).

Six of 20 patients (30%) with a diagnosis of UA expressed very high telomerase activity in comparison with the remaining 14 patients (respectively 2,194, range 806 to 3,696; and 115, range 20.5 to 390). Interestingly, high telomerase UA patients were different from low telomerase UA patients only in the time interval from the last anginal episode and consequent PMN sampling ($p = 0.003$).

Discussion

Our study demonstrates, for the first time, high telomerase activity in PMN from coronary plaque of patients with UA, but not from patients with SA nor in PMN from peripheral blood. Notably, in patients with UA, the only predictor of telomerase activity in the coronary atherosclerotic plaque was a shorter time interval from symptom onset to PMN collection, supporting a possible role of telomerase reactivation in PMN persistence in the plaque during the early phases of coronary instability. Particularly, in all patients with telomerase activity higher than the highest found in SA, the time interval from last anginal episode to coronary PMN sampling was <40 h.

Therefore, in unstable patients with recent coronary instability, the survival of local activated PMN could be prolonged by telomerase reactivation confined to coronary plaque PMN. This mechanism is likely to exacerbate tissue damage and oxidative stress due to PMN activity and to maintain active the inflammatory process, as neutrophil apoptosis has been identified as one of the key mechanism to switch off inflammation.

Activation status of PMN derived from coronary plaques was not measured for the limited number of cells obtained by our new approach (a minimum of 50 up to 200 neutrophils per slide). However, as telomerase reactivation appears to play a key role in delaying apoptosis and inducing growth of cancer cells (17), this intracellular mechanism could also prolong coronary plaque PMN activity merely by extending their local lifespan.

The observation that high telomerase activity is demonstrable in coronary plaque PMN of a subset of UA patients, in whom the measurements were made within 40 h of the last anginal episode, is open to multiple interpretations. This phenomenon might suggest that high telomerase activity plays a key role in triggering coronary instability and is then lost over time. It might represent, however, an inconstant epiphenomenon.

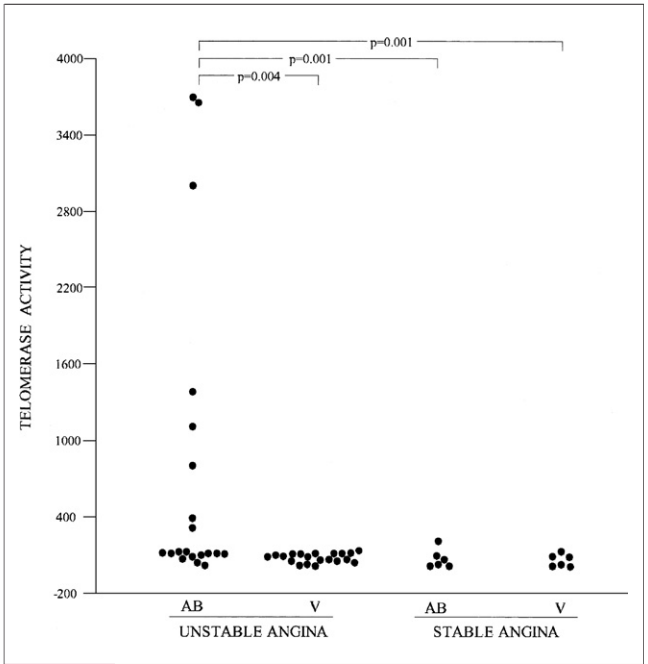

Figure 3 **Distribution of Telomerase Activity**

Telomerase activity in polymorphonuclear neutrophils derived from venous blood (V) or from washing medium of angioplasty balloons (AB) is represented as **dot plots**.

Table 2 Predictors of High Telomerase Activity in Coronary Plaque Neutrophils in Unstable Angina

	Telomerase Activity (n = 20)	
	Univariate Analysis (p Value)	Multivariate Analysis (p Value)
Age	0.65	0.09
Gender	0.40	0.9
Risk factors		
Family history of IHD	0.27	*
Hypercholesterolemia	0.25	*
Diabetes	0.06	0.08
Hypertension	0.25	*
Obesity (BMI >25 kg/m^2)	0.30	*
Smoking (current status)	†	*
Medication		
Beta-blockers	0.20	*
Calcium blockers	0.36	*
ACE inhibitors	0.36	*
Aspirin	0.43	*
Other antiplatelet agents	0.39	*
Lipid-lowering therapy	0.23	*
Hormonal therapy‡	0.93	*
PCI timing§	<0.001	<0.001
Coronary angiographic lesions‖		
Type A	0.47	*
Type B	0.40	*
Type C	0.69	*

*Not performed for p < 0.1 at univariate analysis (age and gender performed because of likely relation to telomerase activity); †not performed on empty groups (smokers in UA group 0%); ‡hormonal therapy is represented by finasteride, a type 2 5alpha-reductase inhibitor; §percutaneous coronary intervention (PCI) timing was defined as time interval between last anginal episode and polymorphonuclear neutrophil sampling; ‖coronary angiographic lesions are classified according to morphology in type A, B, and C (15).

Abbreviations as in Table 1.

Normal human PMN, like other somatic cells, divide a limited number of times before entering a nondividing state called replicative senescence. Telomerase is normally inhibited in these inflammatory cells (18,19). Our study is in keeping with these previous studies, showing that telomerase activity was barely detectable in circulating PMN, but telomerase reactivation in these cells is possible (20) and could represent a way to overcome replicative senescence (5). In our study, the evidence of high telomerase activity in PMN from coronary atherosclerotic plaque suggests a local process leading to intracellular enzyme reactivation resulting in prolonged survival of these inflammatory cells, as typically observed in cell types that retain high proliferative potential (21–23). Indeed, telomere dynamics and changes in telomerase activity are consistent elements associated with changes in proliferative state. Particularly, highly specific correlations and early causal relationships exist between telomerase activation and indefinite cell proliferation (24–26).

We used a novel approach to collect in vivo PMN from coronary atherosclerotic plaques. Our method yielded a PMN population with a purity >90%. This homogenous cell composition is important to minimize noise in TRAP assay for evaluation of telomerase activity. In our study, human telomerase reverse transcriptase ribonucleic acid analysis could not be performed because of the limited number of neutrophils available by direct washing of coronary angioplasty balloons.

Our findings are in keeping with those of Naruko et al. (8) who analyzed neutrophils in coronary culprit stenosis obtained at autopsy in patients who had died of acute myocardial infarction and in atherectomy specimens from SA and UA patients. Neutrophils were detected in 44% of patients with UA and in only 6% of patients with SA.

Recently, reactivation of telomerase activity was observed in vascular smooth muscle cells but not in areas of monocyte-macrophage infiltrations in human atherosclerotic coronary plaque from heart transplant recipients (27). Different mechanism of inflammation in transplanted hearts and in acute coronary syndrome might explain the different pattern of telomerase reactivation in our and in their study.

Garlichs et al. (13) have recently observed a marked delay of circulating PMN apoptosis, in patients with acute coronary syndromes, while, in our study, we failed to find high telomerase activity in circulating PMN; as our findings support the notion that telomerase reactivation of PMN from coronary plaques is specifically related to mechanisms operating at the level of atherosclerotic plaque but not in systemic PMN activation, we hypothesized that in patients with UA different mechanisms are responsible for PMN-delayed apoptosis in coronary plaque milieu and in peripheral blood.

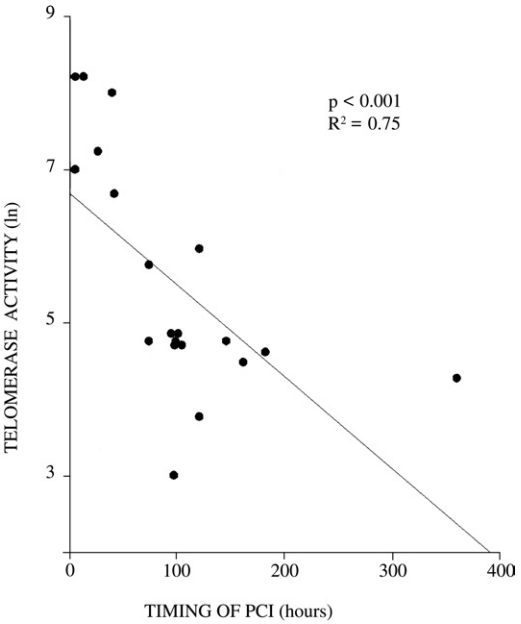

Figure 4 Linear Regression Between Telomerase Activity And Timing of PCI

Among unstable angina patients, a significant inverse correlation was found between telomerase activity (ln: natural logarithm) in polimorphonuclear neutrophils obtained by atherosclerotic plaques and time interval (h) between last anginal episode and polimorphonuclear neutrophils sampling, during percutaneous coronary intervention (PCI).

An important limitation of our study is represented by the precise origin of coronary PMN. Indeed, PMN might theoretically come from coronary thrombus and not necessarily from the atherosclerotic plaque. This limitation, however, does not reduce the importance of our findings as we demonstrate for the first time that a specific reactivation of telomerase occurs only at the local level of the unstable plaque and not in the systemic circulation. Of note, systemic contamination of angioplasty balloons is unlikely, as telomerase activity was always absent in peripheral neutrophils.

A second limitation of our study is represented by the small sample size of SA patients, reflecting our policy of not overtreating with PCI stable patients with a low risk of future events, in agreement with the results of the COURAGE (Clinical Outcomes Utilizing Revascularization and Aggressive DruG Evaluation) study (28).

In a previous study, Biasucci et al. (10) reported that neutrophil activation (analyzed by intracellular myeloperoxidase index) was present in 93% of UA patients but only in 12% of chronic SA patients, demonstrating a large effect size that could be translated to our study. This limitation is attenuated by the intraindividual assessment of telomerase activity from plaque and from systemic circulation PMN, which strengthen the statistical significance of this study.

Conclusions

In patients with UA, but not in patients with SA, significant telomerase activity was detected in the coronary plaque but not in circulating PMN, in particular when PMN were obtained within a few hours of the last anginal episode. These findings suggest local extended lifespan and prolonged activity of these inflammatory cells in the early phase of instability. Because of the toxic potential of PMN, this mechanism may represent a contributory pathway in the pathogenesis of instability. Further studies are warranted in order to establish the precise relationship between telomerase reactivation and neutrophil activity in the unstable coronary plaques.

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Reprint requests and correspondence: Dr. Maria Lucia Narducci, Institute of Cardiology, Largo "A. Gemelli" n.8, 00168 Rome, Italy. E-mail: lianarducci@yahoo.it.

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