Different Quantitative Apoptotic Traits in Coronary Atherosclerotic Plaques From Patients With Stable Angina Pectoris and Acute Coronary Syndromes

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**Background**—Apoptosis in human atherosclerotic coronary plaques possibly causes plaque destabilization by contributing to the weakening and breaking down of the fibrous cap. We tested the hypothesis that apoptosis is quantitatively increased in unstable atherosclerotic plaques.

**Methods and Results**—We analyzed the expression of apoptotic genes such as BAX, CASP1, FAS, FAS L, FOS, MDM2, NFkB2, P53, PCNA, TERT, and XRCC1 in coronary plaques collected with directional coronary atherectomy from 15 patients with stable angina and 15 with acute coronary syndromes without ST elevation (ACS). Total RNA was extracted and cDNA was amplified with a specific set of primers and TaqMan probes. Apoptosis was also revealed by DNA laddering. To clarify the source of mRNAs, we performed in situ reverse transcriptase–polymerase chain reaction coupled with immunocytochemistry and found a substantial overlap between the mRNAs of the above genes and vascular smooth muscle cells. Gene expression analysis showed that the proapoptotic genes (ie, BAX, CASP1, FAS, FAS L, FOS, NFkB2, P53, PCNA) were significantly more expressed (P<0.001) in ACS plaques, whereas the antiapoptotic genes (ie, MDM2, TERT, XRCC1) were more transcribed (P<0.001) in stable angina plaques. Total gDNA gel electrophoresis identified a laddering pattern in the ACS plaques as evidence of end-point apoptosis. Western blotting substantially confirmed the above data.

**Conclusions**—Our findings support the idea that ACS plaques are committed to apoptosis through an established meshwork of gene activation and inactivation, whereas stable angina plaques retain active cell homeostasis and repair mechanisms. (*Circulation. 2004;110:1767-1773.*)

**Key Words:** acute coronary syndromes ▪ angina ▪ apoptosis ▪ atherosclerosis ▪ gene expression
condensation, cell shrinkage, budding, and apoptotic body formation as the first morphological evidence. More recently, considerable progress in molecular biology has provided researchers with various tools for detecting apoptosis such as the terminal Deoxynucleotidyl Transferase-mediated dUTP-biotin nick end labeling technique, agarose gel electrophoresis of extracted DNA, fluorescence dye staining methods, and flow cytometry.\textsuperscript{12}

We analyzed the expression of the most important apoptotic genes (see http://www.ahajournals.org) in atherosclerotic coronary plaques excised from patients undergoing interventional coronary atherectomy for stable angina (SA) or unstable coronary syndromes. We then concentrated on the genes belonging to a well-known and regulated p53 network that is clearly associated with apoptosis.\textsuperscript{13}

The aim of the study was to determine whether it is possible (1) to detect, localize, and quantify the expression of key apoptotic genes in coronary plaques; (2) to assess differences in the expression of apoptotic genes between patients with acute and stable coronary syndromes; and (3) to determine whether differences at the protein level correlate with mRNA expression patterns.

Methods

Patients

The study involved 15 patients with SA (Canadian Cardiovascular Society classes I through III) and 15 with acute coronary syndromes without ST elevation (ACS) who underwent directional coronary atherectomy for a single de novo lesion. Patients were excluded if they had multivessel coronary artery disease, defined as visually assessed >70% diameter stenoses in >1 major coronary artery. All enrolled patients gave informed consent, and the study was approved by the Ethics Committee of Policlinico San Matteo.

Selective coronary arteriography was performed in multiple views with Judkins’ technique. Coronary lesions were treated by directional coronary atherectomy using standard clinical procedures. Briefly, after a guidewire was passed through the lesion, the atherectomy device was positioned across the stenosis, and after inflation of the support balloon, the rotating cutter slowly advanced to cut the protruding atherosclerotic lesion and to collect the plaque in the collection chamber at the tip.

To obtain maximum lesion debulking, the operator made the initial cuts toward the eccentricity; subsequent cuts were made in the other plaque-bearing quadrants. After 4 cuts, the angiographic result was established, and the positive areas are expressed as percentages of the total plaque area.

Plaque Processing and Nucleic Acid Extraction

After atherectomy, half of each plaque was resuspended in 0.5 mL of 2X lysis buffer (Applied Biosystems) and then immediately frozen in liquid nitrogen; the rest was processed for immunohistological and DNA ladder procedures (see In Situ PCR below). Total RNA and gDNA extractions were made by use of the ABI PRISM 6100 platform according to the manufacturer’s instructions, and the first eluate washes were kept for protein analysis (see below). After extraction, each DNA and RNA sample was resuspended in a final volume of 100 μL elution buffer.

Reverse Transcription

Total RNA (100 ng) was reverse transcribed using a high-capacity cDNA Archive Kit (Applied Biosystems). For all further analyses, the cDNA was diluted 1:20 in water.

Quantitative Polymerase Chain Reaction for Gene Expression

For the screened apoptotic genes (listed at http://www.ahajournals.org/miambioexpress/cgi-bin/mx.cgi), we used Assay on Demand FAM-MGB-labeled probes (available at http://www.home.applied-biosystems.com/catalog). We always coamplified the 18S reference gene (VIC-MGB-labeled probe; PN 4319413E) with the target genes in the same wells to normalize the different amounts of total RNA in the different wells and their reverse transcription (RT) reaction efficiencies. RT–polymerase chain reaction (PCR) was performed with the automated ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The cDNA samples were also tested for gDNA contamination, the overall level of which was ≤0.005%.

Threshold Cycle (C\textsc{t}) and the Comparative Method (2\textsuperscript{ΔΔC\textsc{t}})

The differentially expressed genes were quantified by use of the comparative C\textsc{t} method.\textsuperscript{14} The human heart cDNA pool (Stratagene, PN 735012) was required only as a normalization step, and other cDNA could have been used. Data are expressed as the median-fold differences between the SA and ACS plaques.

Data Analysis

The SA and ACS groups were compared by means of 1-way ANOVA, followed by Duncan’s multiple-range test. For differences between the 2 groups, Student’s t test was used when appropriate. Values of P<0.001 were considered statistically significant. The σ values of every target gene in each gene panel between the SA and ACS groups (which were always <0.05%) support the reliability of the generated data. To assess the reproducibility of the method, we calculated the coefficient of variation for each gene, which was always ≤0.67%.

In Situ RT-PCR

The second half of the plaque fragments (see Plaque Processing and Nucleic Acid Extraction above) was fixed in 4% formalin overnight. After water rinsing, the pieces were dewaxed by xylene treatment at room temperature for 2 hours. The slides were then rehydrated with ethanol 100%, 95%, 80%, and 70% and PBS (10 minutes each); passed through Trion X-100 0.01% in PBS (1 minute and 30 seconds) and PBS alone (2 minutes); treated with 0.05% Proteinase K (Roche Molecular Systems) in Tris 20 mmol/L and Tween 20 0.5% for 30 minutes at 37°C; and then transferred to a microwave oven to boil for 30 seconds.

Specific primers for the in situ RT amplification were designed as described previously\textsuperscript{15} (sequences available on request).

After the in situ RT-PCR, the samples were immunostained with a monoclonal anti-VSMC antibody (Sigma-Genosys) and anti-sheep FITC-labeled antibody (DAKO) according to the manufacturers’ instructions. The sections were then counterstained with DAPI (Sigma) with 0.02 mg/mL PBS for 15 minutes at 37°C, thus leading to fluorescent blue nuclei. The slides were directly analyzed under an epifluorescence microscope (Olympus Provis) equipped with a thermoelectronically cooled charge-coupled camera (Photometrics CH350). SMC actin was immunohistochemically quantified by means of computer-assisted color image analysis (BIOQUANT, R&M Biometrics). A color threshold mask for the positive was established, and the positive areas are expressed as percentages of the total plaque area.

DNA Electrophoresis

Total gDNA was extracted as described above, and its fragmentation was analyzed by means of 1.8% agarose gel ethidium bromide–stained electrophoresis; 400 ng of gDNA for each plaque was loaded and run for 10 minutes at 100 V and then 40 minutes at 70 V. Data were acquired and analyzed with GelDoc equipment (BioRad).
Supplementary Information for Gene Expression

Additional data are available in the online-only Data Supplement at http://www.circulationaha.org.

Protein Analysis: Western Blotting

The proteins from the 30 plaques were harvested from the elution washes performed during RNA/gDNA extraction. The lysates were resuspended in 100 μL RIPA lysis buffer, transferred to tubes, rocked for 20 minutes, and then spun at 12 000 rpm for 10 minutes. The supernatant was stored until use. Protein content was estimated with the Pierce BCA method. Extracts containing 10 to 40 μg protein were boiled in 2× SDS sample loading buffer, resolved by SDS-PAGE, and blotted onto 0.2-μm-pore Immun-Blot PVDF membranes (BioRad) in Towbin buffer without methanol at 200 mA for 5 hours. The membranes were blocked for 1 hour in TBS containing 5% skimmed milk, and Western blots were performed with primary antibodies (1:250 Sigma-Genosys) and HRP-conjugated anti-rat or anti-rabbit IgG (1:100000; Sigma) in TBS plus Tween 20.0.2%. Chemiluminescence was revealed, and prestained standard protein markers (BioRad) were used as molecular markers.

Results

Quantitative PCR

We analyzed the expression of 268 genes involved in apoptosis (for a complete list, see http://http://www.ebi.ac.uk/miamexpress/cgi-bin/mx.cgi, protocol ARRAYPRTCL2527) in 15 SA and 15 ACS plaques collected by means of atherectomy. Data were normalized using commercial total heart cDNA and compared between the 2 groups. Figure 1A shows the gene expression patterns. The trend of the expression of apoptosis was substantially similar in the SA and ACS plaques (red dots; correlation values: \( y = 0.9586x + 0.097; r^2 = 0.9837 \)), but there were statistically significant differences (P<0.001) for

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<th>Time-Fold Overexpression of Proapoptotic and Antiapoptotic Genes in the ACS and SA Plaques</th>
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p53 is 600 times more expressed in ACS than in SA plaques.
some genes in the ACS (blue dots) and SA (yellow dots) plaques. We therefore concentrated on the genes with a regulation that was markedly different between the 2 groups.

Figure 1B and the Table show that proapoptotic gene expression was statistically higher in the ACS plaques ($P<0.001$) and that antiapoptotic gene expression was higher in the SA plaques. In particular, the expression of p53 ($TP53$), a key player in the apoptotic process caused by DNA damage, was 600 times higher in the ACS plaques, whereas its inhibitor $MDM2$ (murine double minute 2), a ubiquitin ligase that targets p53 for proteasome destruction, was 8 times more expressed in the SA plaques. Active p53 transactivates proapoptotic genes, including $BAX$ (15.7 times higher in the ACS plaques) to promote full apoptosis. The expression of the $CASP1$ downstream player in the apoptotic cascade was 5.7 times higher in the ACS plaques. p53 can also be activated by a decreased DNA excision repair system ($XRCC$ genes) via the ARF pathway, and the expression of $XRCC1$ was 7.4 times higher in the ACS plaques.

$hTERT$, which ensures chromosome stability, was 4.2 times more expressed in the SA plaques, whereas the ACS plaques had a 2.3-times-higher expression of $FAS$ (a typical death receptor, along with the TNF-R), and a 19-times-higher expression of its ligand ($FAS-L$).

In Situ RT-PCR and Immunocytochemistry

On the basis of the in vitro results, we decided to analyze whether the differentially expressed mRNAs colocalized with specific VSMC lineages in SA and ACS plaques. These cells were selected because of their involvement with macrophages and T lymphocytes in atherosclerotic lesions and because of their established involvement in apoptosis initiation and progression.

The total number of VSMCs was counted over a thickness of 100 μm (20 slices) for each plaque; the specific antigen (SMC α-actin) was detected in 18% of the cells in the SA plaques and 53% of the cells in the ACS plaques ($P<0.001$). Moreover, the level of the subfraction of VSMCs expressing p53 (evaluated by in situ RT-PCR) was significantly higher in the ACS than the SA plaques (83% versus 32%; $P<0.001$).

Figure 2A shows the double labeling for the colocalization of cell types (VSMCs) and mRNAs as revealed by immunocytochemistry reactions using Genosys antibodies (green fibers) and in situ RT-PCR to detect p53 mRNAs (purple grains) in an ACS plaque ($\times 80$). To provide quantitative data based on specific over/underexpression of the gene and/or protein stabilization, Figure 2B shows the average cellularity counts over a thickness of 100 μm in the 30 plaques (roughly 9000 cells per plaque). The y axis represents the total cell number; the blocks with inner vertical lines represent number of VSMCs (1620±20 in SA and 4400±32 in ACS plaques); and the solid blocks represent the subfraction of VSMCs expressing p53 (518±12 in SA and 3652±18 in ACS plaques).

DNA Fragmentation

Figure 3 shows the typical ladder pattern of fragmented gDNA in the ACS plaques (400 ng/well; upper right lanes), whereas little ladder was detectable in the SA plaque or control lanes (cultured fibroblasts). The main factor responsible for this digested pattern is apoptosis-induced DNase $\gamma$.

Protein Analysis

To confirm and integrate our mRNA data, we performed Western blotting of 30 plaque lysates; we report here the data for SA plaque 12 and ACS plaque 15. Figure 4A shows the immunoblotting staining reactions for native p53 and BSA (loading control). The immunostaining in the
ACS plaque was 123 times greater than in the SA plaque ($P<0.001$), but as shown in Figure 4B, the level of MDM2 was 6 times higher in the SA plaque. To analyze further the presence of modified p53, we exposed the plaque lysates to an anti-acetylated-p53 (AcLys$_{392}$) (Figure 4C) and an anti-phospho-p53 (pSer$_{392}$) (Figure 4D). In both cases, the response of the ACS plaque was greater than that of the SA plaque (4 and 12 times more, respectively; $P<0.001$). The AcLys$_{392}$ modification is induced by many stressing agents (ie, ionizing and/or ultraviolet radiations, hypoxia, chemicals, mechanical stress), whereas the pSer$_{392}$ reaction is specifically UV induced.$^{16}$

Figure 4E shows the TaqMan analysis of the protein expression patterns of the same genes in the 2 plaque groups (blue for ACS, yellow for SA), which substantially reflected the relative abundance of the gene mRNAs. We found 82% concordance with $P<0.01$ ($PCNA$ and $FOS$ were not significant), 91% with $P=0.01$, and 100% with $P=0.05$.

**Discussion**

This study showed that apoptosis is present in atherectomy-derived coronary lesions associated with both SA and unstable angina. Using various techniques, we highlighted the presence of proapoptotic messengers within specific cell types, detected quantitative differences in their expression patterns, and visualized their final impact on cell destiny.

These gene expression data indicated that the qualitative expression patterns of key apoptotic genes were similar in the
atherosclerotic coronary plaques of SA and ACS patients, but their quantitative traits were very different. Using morphological data, Bauriedel et al. suggested that the vulnerability and rupture observed in ACS plaques and the stability observed in SA plaques may be due to different apoptotic patterns and mechanisms. However, our findings show that, although the same genes are expressed in both, proapoptotic genes are more active in ACS plaques and antiapoptotic genes are more transcribed in SA plaques. Moreover, our data support the idea that ACS plaques are committed to programmed cell death via an established meshwork of gene activation and inactivation, whereas SA plaques retain active cell homeostasis and repair mechanisms.

It has previously been shown that a close link exists between inflammation and apoptosis in atherosclerotic plaques, and our data support these findings. In particular, ACS plaques showed a higher expression of FAS, a major component of the intrinsic apoptosis-activating pathway, and FAS-mediated apoptosis is a characteristic of atherosclerotic plaque instability. Interestingly, the FAS ligand (FAS-L) was much more expressed than its receptor in ACS plaques. This imbalance between the receptor and its ligand could be due to the presence of a paracrine loop activating suicide of cells involved in atherosclerotic lesions (such as VSMCs and macrophages), as in the case of T cells.

Because in vitro studies have shown that p53 regulates apoptosis in cultured VSMCs, we concentrated on our in situ RT-PCR and immunohistochemistry experiments on VSMCs and p53. It has been suggested that VSMC proliferation may be involved in the pathogenesis of atherosclerosis, and it is true that VSMCs removed from plaques show higher levels of spontaneous apoptosis than normal VSMCs. We hypothesize that in some settings (i.e., SA plaques) maintaining cells rather than eliminating them by apoptosis may be advantageous (like in ACS plaques).

Figure 2 shows that p53 mRNAs and VSMC-specific antigens were colocalized in 80% of the hits, although the immunohistochemical detection of p53 has led to discrepant results. p53 protein staining of tissue sections is critical; it not only depends on tissue fixation but also requires thorough antigen retrieval. We obtained stable and reproducible results using formalin-fixed and paraffin-embedded tissues by applying in situ RT-PCR, i.e., the direct detection of p53 mRNAs.

In addition to studying gene expression using 5′ nuclease assay and in situ RT-PCR coupled with immunocytochemistry, we also revealed apoptosis using gel electrophoresis to detect the presence of DNA fragmentation (Figure 4). This process leads to a typical cleavage pattern of 180 to 200 bp triggered by a subset of apoptosis-dependent DNases (DNase γ). Our data clearly show this pattern in ACS plaques, whereas the gDNA in SA plaques retains its high molecular weight as in normal cells. The DNA breakdown in ACS plaques is well regulated and does not produce the gel smear that may be induced by tissue necrosis, whereas the XRCC1 repair system (for AP sites) still ensures viable DNA in SA plaques.

We concentrated on p53, one of the pivotal proteins involved in apoptosis. The p53 network is normally "off" and is activated only when cells are stressed or damaged. However, because it is a key stress-response protein, its rapid activation is essential, and both goals are achieved by its rapid turnover. Furthermore, its abundance and ability to bind specific target sequences seem to be regulated predominantly by posttranscriptional mechanisms. MDM2 is a key regulator of p53 stability, but the presence of mutations/modifications in various amino acids residues can compromise this activity. Mutations in Lys370 (acetylation) show little ubiquitination even at higher MDM2 levels, whereas mutations/modifications in Ser32 (phosphorylation) increase the tetramerizing ability of p53 and therefore its transcriptional activity, despite high MDM2 levels. We found that p53 protein levels were higher in ACS plaques and correlated with lower MDM2 levels and that modified p53 (acLys370 and pSer32) could "escape" MDM2-feedback negative regulation. In relation to the other proteins involved in the p53 network, we found a good relationship between gene expression and Western blot data, indicating an analytical tool (i.e., gene expression) that could integrate the phenotypical assessment of atherosclerotic lesions offered by protein profiling.

In conclusion, our results support the idea that mechanisms underlying cell homeostasis and repair are still active and more balanced in SA plaques, whereas ACS plaques may be characterized by an unbalanced commitment to programmed cell death as a result of development of preferential pathways activating specific apoptotic genes. Nevertheless, apoptosis may play a significant role in plaque destabilization and rupture by magnifying local inflammatory responses and thrombotic events. This supports the idea that keeping cells rather than eliminating them by apoptosis may be relevant for the organism. Further studies are needed to improve our understanding of the molecular basis of apoptosis and inflammation in ex vivo plaque specimens and cultured cell lines such as VSMCs and to clarify which mechanisms lead to apoptosis in some cell types and to premature senescence in others even if p53 network activation is similar.

References


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