

## ORIGINAL ARTICLE

## Evidence that protease activated receptor 2 expression is enhanced in human coronary atherosclerotic lesions

C Napoli, F de Nigris, J L Wallace, M D Hollenberg, G Tajana, G De Rosa, V Sica, G Cirino

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See end of article for authors' affiliations

Correspondence to: Dr C Napoli, Department of Clinical Pathology and Medicine, University of Naples, PO Box 80128, Naples, Italy; clunap@tin.it

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**Aim:** To investigate protease activated receptor 2 (PAR-2) expression in human coronary atherosclerotic lesions because PAR-2 is involved in the modulation of inflammatory events and vascular function.**Methods:** An immunohistochemical analysis was performed on serial arterial sections, using the following antibodies: MDA2, a murine monoclonal antibody against malondialdehyde lysine epitopes of oxidised low density lipoprotein (oxLDL); HAM-56, a monoclonal antibody against human macrophages/foam cells; B5, a rabbit polyclonal antibody against PAR-2; and SAM11, a mouse monoclonal antibody against human PAR-2. Sections containing at least one lesion showing substantial immunostaining were counted as positive, and results were expressed as per cent of all sections of the same artery.**Results:** PAR-2 expression was enhanced in human coronary atherosclerotic lesions. This phenomenon correlated with an increase in oxLDL epitopes in the coronary artery.**Conclusion:** This study shows for the first time that PAR-2 expression is enhanced in human coronary atherosclerotic lesions, and suggests that PAR-2 dependent cellular trafficking may be one of the regulatory signalling responses to vascular injury. Further pharmacological studies will establish whether modulation (and in which direction) of PAR-2 represents a possible therapeutic target for controlling the vascular response to injury.

Thrombin is one of the key molecules involved in the development of vascular diseases. Thrombin does not only serve as a coagulation factor, but it also exerts cellular effects by activating protease (proteinase) activated receptors (PARs), a family of seven transmembrane G protein coupled receptors activated by proteolytic cleavage.<sup>1</sup> The pathophysiological role in vivo of protease activated receptor 2 (PAR-2) remains poorly understood.<sup>1,2</sup> PARs are vascular sensors for signalling of the trypsin-like coagulation serine proteases.<sup>1,2</sup> PAR-2 is expressed in arteries<sup>3–7</sup> and in the heart,<sup>8–10</sup> and plays a role in the regulation of vascular tone<sup>4,7,11–14</sup> and tissue inflammation.<sup>6–8,10,11,15–17</sup> Endothelial cells have a primary role in mediating the vascular effects of PARs under physiological conditions, whereas PARs can be induced in smooth muscle cells under pathological conditions, and therefore play a more pathophysiological role.

“Targeting inflammatory cell signalling events of the coagulation system may become an important aspect of efforts to improve antiatherothrombotic treatment”

Because proteases are activated during pathological states such as haemorrhage, tissue damage, and inflammation, PARs have been suggested to play a crucial role in the development of functional and structural abnormalities in vascular lesions. Indeed, inflammation is a well established component of the vulnerable atherosclerotic plaque.<sup>18</sup> Targeting inflammatory cell signalling events of the coagulation system may become an important aspect of efforts to improve antiatherothrombotic treatment. Because we hypothesised that PAR-2 activation is involved in inflammatory and injury response events,<sup>1</sup> here, we attempt to verify whether PAR-2 plays a role in human coronary atherosclerotic lesions.

## METHODS

## Human subjects

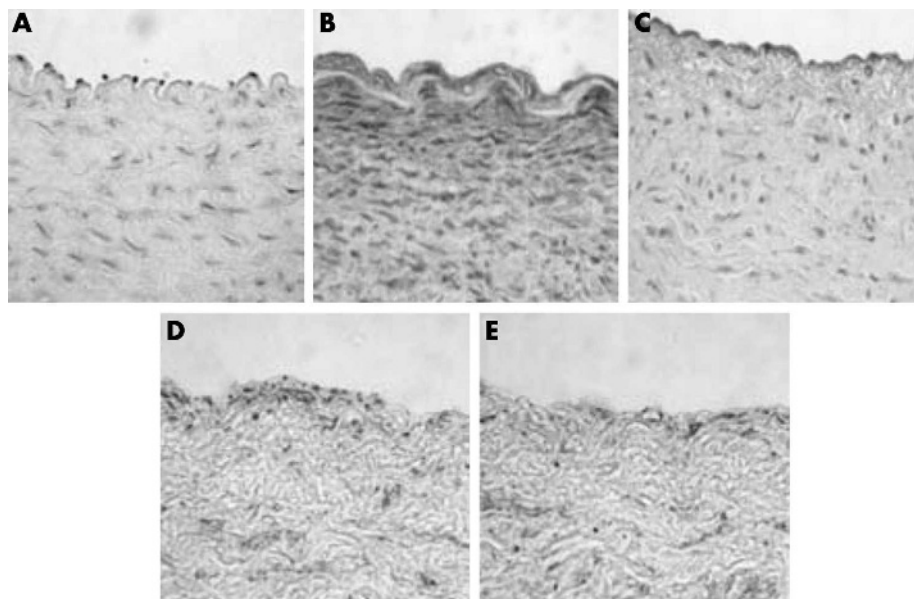
Arteries were collected from adult (mean age, 41.8 years; SD, 5.6; n = 8) and elderly (mean age, 70.5 years; SD, 5.1;

n = 6) men subjected to necropsy at the University of Naples, who had died in traumatic accidents. They were included in our study only if they were men (to avoid sex related differences) and had no classic risk factors for atherosclerosis (family history of coronary heart disease, diabetes, smoking, hypertension, or dislipidaemia), which were assessed by detailed medical history of clinical records, as described previously.<sup>19</sup> Our study protocol was approved by the local human ethical committee.

## Preparation of arterial sections, histological analysis, and immunohistochemical analysis

Using a stereo microscope, the entire left anterior descending and the right coronary arteries were dissected from each patient, cut open, washed thoroughly with cold sterile phosphate buffered saline (PBS), and placed in ice cold PBS containing 50µM butylated hydroxytoluene, 0.001% aprotinin, 50mM EDTA, 1µM deferoxamine, and 0.008% chloramphenicol, equilibrated with nitrogen. One of these segments was immersed in ornithine transcarbamoylase, flash frozen in liquid nitrogen, and 30–40 sections (7 µm thick) were prepared for computerised morphometric determination of lipid rich lesions by oil red O staining, as described previously.<sup>19–21</sup> Next, we determined the cumulative area of all lipid accumulations for each section. Alcian blue staining was used to evaluate nuclear smooth muscle cell density.<sup>19</sup> Another arterial segment was fixed in buffered 10% formalin, paraffin wax embedded, and 12–15 serial sections (5–7 µm thick) were prepared for immunohistochemistry, as described previously.<sup>19–21</sup> Alternate serial sections were immunostained with: (1) MDA2, a murine monoclonal antibody against malondialdehyde lysine epitopes of oxidised low density lipoprotein (oxLDL)<sup>19–21</sup>; (2) HAM-56, a monoclonal antibody against human macrophages/foam cells (Axcel Accurate, Westbury, New York, USA)<sup>19–21</sup>; (3) B5, a

**Abbreviations:** oxLDL, oxidised low density lipoprotein; PAR-2, protease activated receptor 2; PBS, phosphate buffered saline



**Figure 1** Representative immunostaining for protease activated receptor 2 (PAR-2; B5 antibody, 1/250 to 1/500 dilution) of human coronary arteries. (A) Control healthy tissue (oil red O negative staining) of coronary artery of an adult subject; (B) PAR-2 positive immunostaining in an early transitional coronary lesion of an adult subject; (C) faint immunostaining in the subendothelial space from a calcific coronary lesion in an elderly subject. Colocalisation in serial sections of a coronary lesion of an adult subject immunostained with (D) B5 and (E) MDA-2 antibodies. Original magnification,  $\times 640$ .

rabbit polyclonal antibody against PAR-2<sup>8 10 22</sup>; and (4) SAM11, a mouse monoclonal antibody against human PAR-2 (Zymed Laboratory, San Francisco, California, USA). All antibodies were used at a dilution between 1/500 and 1/100. Unless otherwise specified, epitopes recognised by the primary antibody were detected by an avidin–biotin–peroxidase method.<sup>19–21</sup> Immunohistochemical colocalisation using two antibodies was performed and analysed by computer assisted imaging software.<sup>19–21</sup>

### Statistical analysis

Results were analysed by one way ANOVA, followed by Bonferroni's, and a  $p < 0.05$  was considered to be significant. Numerical data obtained from immunohistochemistry were analysed for mean, variance, SD, kurtosis, and skew. Correlations between the results were also evaluated by linear regression analysis. All data were analysed by SPSS statistical package (SPSS Inc, San Diego, California, USA).

## RESULTS

### Extent and maturity of atherosclerotic lesions

Based on computer assisted imaging analysis of histological oil red O stained sections, we divided coronary atherosclerotic lesions into early class I lesions (fatty streaks), class II lesions (fibrous lesions), and advanced class III (fibroatheromatous lesions) and class IV (calcific lesions) lesions. The mean (SD) lumen stenosis of the lesions was 45% (6%) in adults and 65% (8%) in elderly men ( $p < 0.05$ ). Mean intimal thickness increased with age in coronary arteries ( $p < 0.05$ ). The nuclear density of the smooth muscle cells in the media, as an index of the maturity of the lesions,<sup>19 20</sup> varied with the stage of the lesions, and ranged from a mean (SD) of 752 (123) smooth muscle cell nuclei/mm<sup>2</sup> in adult coronary arteries to 1233 (227) in those from elderly men ( $p < 0.01$ ). Data were not normally distributed in the lesions (cumulative skew of 2.04) and were therefore analysed by the Wilcoxon test.

### Immunohistochemistry

Paraffin wax embedded serial sections of coronary arteries from the study population were immunostained and assessed for the intimal presence of oxLDL, macrophage derived foam

cells, and PAR-2. Sections containing at least one lesion showing positive immunostaining were counted as positive, and results were expressed as per cent of all sections of the same artery, as described previously.<sup>19–21</sup> The early and transitional lesions (class I and II) contained significantly higher staining for PAR-2 than those of class IV (fig 1A–E). PAR-2 immunostaining was typically seen at the subendothelial space (table 1). Results obtained with both antibodies were highly correlated when all the lesions were considered ( $r = 0.72$ ;  $p < 0.001$ ).

Computer assisted imaging analysis of colocalisation (fig 1D, E) showed a positive immunohistochemical correlation between oxLDL and PAR-2 (B5 antibody) in class I and II lesions ( $r = 0.48$  and  $0.41$ , respectively;  $p < 0.01$ ). The correlation between PAR-2 and oxLDL was also significant in class III lesions using the SAM11 antibody ( $r = 0.51$ ;  $p < 0.01$ ). Thus, as vascular inflammation increased the correlation between oxLDL and PAR-2 increased—the correlation between oxLDL and PAR-2 was stronger in class III lesions than in class I and II lesions. However, there were no correlations in class IV lesions ( $p = \text{NS}$  for both B5 and SAM11 antibodies), probably because multiple inflammatory pathways are simultaneously activated in such advanced atherosclerotic lesions. These immunostaining data reflected a simultaneous increase for both antibodies in the same areas and not increased numbers of nuclei in serial sections.

## DISCUSSION

This is the first evidence that the PAR-2 protein is present in early coronary atherosclerotic lesions in humans without confounding risk factors for atherosclerosis. We have shown that calcific atherosclerotic lesions in coronary arteries (class IV) have reduced PAR-2 expression. The results are particularly consistent because the immunohistochemical data from the two independently prepared antisera (B5 and SAM11 antibodies) were the same.

The details of the complex framework of pathological processes leading to the formation of atherosclerotic lesions are well documented. Atherosclerosis based diseases are the leading cause of death and disability in the world. Because classic risk factors may increase per se the degree of coronary atherosclerosis and vascular inflammation, here, we selected prospectively only men who did not have the classic risk factors for the disease.<sup>19</sup> We showed that the presence of

**Table 1** Number of sections positive for PAR-2 staining (% of positive sections with two independent antibodies) in relation to the stage of the atherosclerotic lesions

	Stage of lesion			
	Class I	Class II	Class III	Class IV
PAR-2 (B2 antibody)	56% (8%)	69% (11%)	43% (12%)	25% (9%)*
PAR-2 (SAM11 antibody)	43% (10%)	58% (14%)	46% (14%)	28% (11%)†‡

We examined 72 class I and class II atherosclerotic lesions, 49 from arterial serial sections of 8 adults and 23 from arterial serial sections of 6 elderly men. Moreover, we studied 96 class III and class IV atherosclerotic lesions, 43 from arterial serial sections of 8 adults and 53 from arterial serial sections of 6 elderly men. \* $p < 0.01$  v class I and II lesions; † $p < 0.01$  v class II lesions; ‡ $p < 0.05$  v class I lesions. PAR-2, protease activated receptor 2.

PAR-2 protein in lesions was significantly related to oxidation specific epitopes of oxLDL (MDA-2 antibody) in class I, II, and III lesions. Clearly, the relative contribution of these potential mechanisms to atherogenesis and vascular inflammation, the functional relevance of changes in PAR-2 expression, and their interactions need to be investigated in experimental models of the disease, rather than in post-mortem tissues. Although this does not represent a causal association, differences in intracellular and extracellular oxidative processes could exist in human coronary arteries, which in turn may affect PAR-2 dependent gene expression. Obviously, it is well recognised that atherogenesis is a complex inflammatory disease.<sup>18</sup> It is therefore possible that a broad spectrum of inflammatory and oxidation sensitive genes could be activated,<sup>23, 24</sup> especially in class II and III lesions. Thus, the mechanism involving PAR-2 activation and oxidation sensitive pathways in the arterial wall could perpetuate the condition of "oxidative stress and inflammation". We cannot establish whether increased in situ PAR-2 activity is a primary phenomenon or whether it is secondary to the atherogenic and/or inflammatory process; however, when atherogenesis affects coronary arteries, this may induce activation of PAR-2 in a vicious cycle. Further studies are also needed to establish whether the changes in protein concentrations are the result of increased gene expression or stabilisation of the protein.

Previous data also indicate that PAR-2 plays an important role in myocardial ischaemia-reperfusion injury,<sup>8, 10</sup> an inflammatory state in which a large amount of oxygen radicals are generated at reflow. This further suggests a broad spectrum of oxidation sensitive PAR-2 activities in the cardiovascular system. Thus, the PAR-2 dependent network may play a pivotal role in the inflammatory response and oxidative injury to tissue.<sup>1, 2</sup> In this context, vascular inflammation linked to atherosclerosis may represent an additional scenario for the action of PAR-2.

"We showed that the presence of protease activated receptor 2 in lesions was significantly related to oxidation specific epitopes of oxidised low density lipoprotein (MDA-2 antibody) in class I, II, and III lesions"

Previous data in other models,<sup>1, 8, 10, 11, 13</sup> recent data on the angiogenesis promoting effects of PAR-2,<sup>25, 26</sup> and our results indicate that PAR-2 may not necessarily play a pathological role in vascular inflammation. It could reflect a more complex pathophysiological situation, where arteries try to counter-balance the vascular damage caused by atherogenesis by inducing a protective response. In line with this assumption, bacterial proteinases activate PAR-2 on neutrophils,<sup>27</sup> suggesting that PAR-2 may constitute one of the first protective mechanisms that signal invasion of bacterial pathogens by activating a primary inflammatory response. This last consideration might also fit in with the hypothesis that

### Take home messages

- We show for the first time that protease activated receptor 2 (PAR-2) expression is enhanced in human coronary atherosclerotic lesions from humans with no classic risk factors for atherosclerosis
- PAR-2 dependent cellular trafficking may be one of the regulatory signalling responses to vascular injury
- Further pharmacological studies will establish whether modulation of PAR-2 represents a possible therapeutic target for controlling the vascular response to injury

infection and inflammation trigger acute coronary syndromes.<sup>18, 28</sup> Once it is established whether PAR-2 expression is a primary or a secondary response and whether this expression is harmful or just a compensatory mechanism, inducing a protective response, modulation of PAR-2 may represent a possible therapeutic target for controlling vascular response to injury.

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### Authors' affiliations

**C Napoli, V Sica**, Department of Clinical Pathology and Medicine, University of Naples, Naples, 80128 Italy

**F de Nigris, G Tajana**, Department of Pharmacological Sciences, University of Salerno, Salerno, 84084 Italy

**G De Rosa**, Department of Human Pathology, University of Naples, 80131 Italy

**J L Wallace, M D Hollenberg**, Department of Pharmacology and Therapeutics, University of Calgary, Alberta, T2N 4N1 Canada

**G Cirino**, Department of Experimental Pharmacology, University of Naples, 80131 Italy

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