Expression of inducible nitric oxide synthase in human coronary atherosclerotic plaque

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Abstract

Objective: Macrophages in atherosclerotic plaque may express the inducible isoform of NO synthase (iNOS), which produces large amounts of NO. On one hand, the production of NO can be protective by its vasodilatory, antiaggregant and antiproliferative effects. On the other hand, the formation of peroxynitrite from NO may favour vasospasm and thrombogenesis. In this study, we investigated whether iNOS is present in human coronary atherosclerotic plaque, and we correlated these data with the clinical instability of the patients.

Methods: Fragments were retrieved by coronary atherectomy from 24 patients with unstable angina and 12 patients with stable angina. The presence of macrophages, and the production of TNFα, iNOS and nitrotyrosine were detected by immunocytochemistry.

Results: Macrophage clusters were found in 67% of stable patients and 87% of patients with unstable angina (NS). TNFα was expressed in about 50% of cases in both groups. iNOS was not expressed in fragments from stable patients but was found in macrophages from 58% of unstable patients (P < 0.001). The expression of iNOS was associated with the presence of nitrotyrosine residues, a marker of peroxynitrite formation. Expression of iNOS was correlated both with complaints of angina at rest (P < 0.05) and with the presence of thrombus at morphological examination (P < 0.001).

Conclusion: The expression of iNOS may be induced in human coronary atherosclerotic plaque and is associated with different factors of instability. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Atherosclerosis; Cytokines; Macrophage; Nitric oxide; Unstable angina

Recent investigations have underlined the importance of macrophages in plaque instability [1,2]. Activated macrophages produce cytokines [3], which in turn trigger the expression of inducible enzymes, such as the inducible isoform of nitric oxide synthase (iNOS) [4,5]. This enzymatic induction leads to a sustained production of NO which corresponds to approximately 100-fold the amount produced by the constitutive endothelial NOS (EcNOS) [6]. Although the basal rate of NO production by EcNOS protects against leukocyte adhesion, platelet aggregation and vasoconstriction, the high amount of NO produced by iNOS may interfere with plaque stability through different potential mechanisms [7]. Some authors consider that iNOS activity in the plaque is deleterious, due to the formation of peroxynitrite [8–12] – the product of NO and superoxide – which enhances platelet adhesion and aggregation [13,14], induces vascular hyperreactivity [15–18], and alters various proteins by nitration of their tyrosine residues [19,20]. Other studies show that large amounts of NO may be beneficial by favouring vasodilation and by inhibiting cellular proliferation in the plaque [21,22].

The role of iNOS in the pathogenesis of atherosclerotic plaque has been mainly investigated in experimental lesions from animal models, but recent studies have shown that iNOS may be expressed in the human atherosclerotic plaque as well [23,24]. However, it is not known whether...
iNOS expression in coronary atherosclerosis is only a surrogate of macrophage infiltration or whether such induction may be regarded as a player in the pathophysiology of plaque instability. Our aim was therefore to investigate the induction of iNOS in coronary plaque retrieved by directional coronary atherectomy (DCA) from patients with symptomatic angina and to compare these morphological findings with the clinical presentation of patients.

1. Patients and methods

1.1. Classification of patients

A total of 36 patients were included in the study, of whom 12 had chronic stable angina and 24 had unstable angina. Patients were included when no prior intervention had been performed, and when the culprit lesion was clearly identifiable at angiography, greater than 50% stenotic in diameter and successfully treated percutaneously by DCA [25]. The angiographic morphology of coronary stenosis was classified as previously described [26,27]. Quantitative coronary angiography (QCA) [28,29] of the culprit lesion was performed prior to DCA, after injection of intracoronary isosorbide dinitrate (1 to 2 mg). The angiograms were not suitable for QCA in two patients. The reference diameter, the minimal luminal diameter (MLD) and the percentage of diameter stenosis were measured. The clinical records were analyzed by two independent observers unaware of the results of the pathological examinations and the patients with unstable angina were then classified according to Braunwald [30]. This classification groups the patients according to both the severity of symptoms (I = stress angina, II = subacute rest angina, III = acute rest angina) and the circumstances of angina (A = extracardiac cause, B = primary cardiac cause, C = post-infarct angina). Patients with unstable angina after previous bypass surgery or percutaneous revascularization were not included. Patients were considered to present with stable angina if they did not fulfil any of the Braunwald’s criteria. A positive stress test or other evidence for ischemia was required to justify the coronary intervention. Clear indications permitting patient classification were available in all cases at admission and no post-hoc alterations were made.

1.2. Stainings and morphometry

Atherectomy cuts using the Simpson device followed by complementary balloon dilatation were performed as previously described [29]. The number of fragments (two to five in each case) and the amount of tissue were comparable between groups. The fragments were processed and stained as described previously [31]. The following stainings were used in each case: Hematoxylin Eosin Safran, Periodic Acid Schiff reaction and Phosphotungstic Acid Hematoxylin (PTAH). The percent of surface covered by fibrosis, cellularity and macrophages was measured from the entire surface of the samples by quantitative morphometry, using a 100/100-squares grid on a Visopan planimeter (Reichert) at ×25 magnification. Plaque was considered as predominantly ‘fibrosclerotic’ when more than 75% of the surface was covered by fibrosis (dense extracellular protein deposition). Plaque was considered as ‘cellular’ when more than 10% of the surface was covered by cellular hyperplasia, characterized by ‘stellar-shaped fibromyocytes’ (these characteristics are illustrated in Ref. [31]).

1.3. Immunolocalization

Immunocytochemistry was performed on frozen sections. Macrophages were detected with the anti-CD 68 antibody (diluted 1:100; Dako, Denmark). Smooth muscle cells were characterized by the anti-α actin antibody (diluted 1:200; Dako, Denmark). NO synthase was detected using mouse monoclonal antibodies (Transduction Laboratories, U.K.) against both the macrophage-inducible isozyme (anti-MacNOS, dilution 1:100) and the endothelial constitutive isozyme (anti-EcNOS, dilution 1:20). Expression of TNFα was detected with a monoclonal antibody (diluted 1:20, Genzyme). An anti-nitrotyrosine antibody (diluted 1:20, Upstate Biotechnology) was used for the detection of peroxynitrites (produced from the reaction of NO with superoxide). In each case, sections were incubated with the specific antibody for 60 min, then washed for 5 min with phosphate-buffered saline (supplemented with 0.1% BSA) and incubated for 60 min with a polyclonal sheep anti-mouse IgG-biotin conjugate (Boehringer). After washing, streptavidin-coupled peroxidase (diluted 1:400 from 1 mg/ml original concentration) was added. Peroxidase activity was revealed using a commercial kit of 3-amino-9-ethylcarbazol (Dako) with 0.1% H₂O₂. Sections were rinsed in water, counterstained in Mayer’s hematoxylin for 2 min and mounted with an aqueous mounting medium (Dako, Denmark). Quantification of immunopositive cells was made in each case from the entire surface of the fragments, using the same method as described above. Slide examination was performed by two independent observers unaware of the clinical classification. Several controls of labeling specificity were performed. First, to test for non-specific staining by the secondary antibody, the latter was applied in absence of the primary antibody, and no labeling was detected. Second, to test for non-specific fixation of the primary antibodies on human Fcγ receptors, irrelevant class- and type-matched mouse immunoglobulins were used as primary antibodies on control sections and no labeling was observed under these conditions after application of the secondary antibody. The specificity of the anti-macNOS antibody was also verified by incubating sections from each patient with the antibody.
in presence of an excess of antigen from a mouse macrophage lysate (Transduction Laboratories). The lysate was first centrifuged, then resuspended in phosphate-buffered saline. The presence of iNOS in the lysate was checked by Western blotting, using protein markers for molecular mass determination. Some slides were also incubated with an anti-MacNOS mouse polyclonal antibody and its corresponding blocking peptide (seq. 1131–1144, Calbiochem). Incubation with this antibody showed the same labeling as that obtained with the monoclonal antibody, and addition of the blocking peptide totally suppressed the labeling.

1.4. Statistical analysis

Statistically significant differences between groups were calculated using the exact Fischer’s t-test. A value of $P<0.05$ was considered as significant.

2. Results

2.1. Patient characteristics

Patients characteristics are shown on Table 1. No significant differences in demographics, offending vessel and number of diseased coronary arteries were present between patients with stable or unstable angina. The culprit lesion was on the left anterior descending artery in 75% of patients from both groups, and most of patients had one-vessel disease. From the 24 patients with unstable angina, 14 were from class B and ten were from class C. In this last subgroup, DCA was performed 7 days or longer after myocardial infarction. Angiographic characteristics and QCA are shown in Table 2. Mainly, there was no difference in stenosis severity between groups as measured by QCA.

2.2. Histopathological characteristics

The differences in the morphological pattern of plaque fragments from patients with stable or unstable angina were very similar to those described previously [31,32]. As shown on Fig. 1A, the percentage of patients presenting with a fibroelastic lesion was significantly higher in the stable than in the unstable group. The percentage of surface of the fragments covered by fibrosis was $90\pm5\%$ and $65\pm5\%$ in these two groups, respectively ($P<0.01$). At the opposite, the prevalence of proliferative cells and thrombus was significantly higher in patients with unstable than in patients with stable angina (Fig. 1B and C). Media (detected by the presence of internal elastic membrane) was found only in two patients with unstable angina.

<table>
<thead>
<tr>
<th>#</th>
<th>Pat</th>
<th>Male gender</th>
<th>Age</th>
<th>Vessel</th>
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<td>LAD</td>
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</table>

Abbreviations: Pat: patient; vessel: coronary artery in which atherectomy was performed; # dv: number of diseased vessels; class: class of unstable angina according to Braunwald's classification.

2.3. Immunolocalizations

Examples of immunostainings are presented in Fig. 2 and the data are summarized in Fig. 3. Macrophages (Fig. 2A) were found in 67% patients with stable angina and in 87% unstable patients, which was not statistically different (Fig. 3A). However, the surface of the biopsy covered by macrophages was $5.0\pm0.5\%$ of total surface in patients with stable angina and $12\pm1\%$ in patients with unstable angina ($P<0.01$). These morphological differences between stable and unstable plaques are close from those reported in previous atherectomy studies [31,32].

Macrophage activation was assessed by their production of TNFα (Fig. 2C). The presence of TNFα was detected in 58% of cases. The cytokine was found in 5/12 cases (49%) of patients with stable angina and 14/24 cases
Table 2
Morphological and quantitative angiographic characteristics of the coronary lesions

<table>
<thead>
<tr>
<th>Coronary morphology</th>
<th>QCA</th>
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<td>Conc. Ecc. I Ecc. II Thromb.</td>
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<tr>
<td>Stable</td>
<td>(%) (%) (%) (%)</td>
</tr>
<tr>
<td>70 30 0 0</td>
<td>3.58±0.66 1.17±0.45 67±9</td>
</tr>
<tr>
<td>Unstable Class B</td>
<td>38* 38 23 23</td>
</tr>
<tr>
<td>Class C</td>
<td>22* 33 45* 22*</td>
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<tr>
<td>Group I</td>
<td>31* 38 31 23</td>
</tr>
<tr>
<td>Groups II+III</td>
<td>33* 33 33* 22*</td>
</tr>
</tbody>
</table>

Abbreviations: Conc.: concentric lesion; D ref: diameter of reference; D sten: diameter stenosis; Ecc. I: eccentric lesion type I; Ecc. II: eccentric lesion type II; MLD, minimal luminal diameter; QCA: quantitative coronary angiography; Thromb: intracoronary thrombus. * P<0.05 versus corresponding value in patients with stable angina.

(65%) of patients with unstable angina (Fig. 3B; P=NS). When detected, TNFα was always present in macrophages and labeled about 70% of CD 68-positive cells. In 25% of the overall population, TNFα was also found in neointimal smooth muscle cells and labeled about 30% of these cells.

In sections incubated with the anti-iNOS antibody (Fig. 2B), no positive case was found in patients with stable angina, whereas the enzyme was expressed in 14/24 (58%) patients with unstable angina (Fig. 3C; P<0.001 versus patients with stable angina). In each case, the iNOS signal was detected in CD 68-positive macrophages. The iNOS signal was detected in macrophage clusters, rather than in isolated macrophages, and labeled 70% of the total macrophage population. A signal was also detected in the extracellular space around the macrophages (Fig. 2B), which may reflect cell necrosis. Smooth muscle cells within atherosclerotic fragments, detected by the anti-α smooth muscle cell actin antibody (Fig. 4A), were not positive for iNOS (Fig. 4B). PTAH-positive thrombi were not labeled by the anti-iNOS antibody. Labeling with the anti-EcNOS antibody identified the endothelial cells of neovessels arising in the coronary atherectomy fragments, but no labeling was detected in either the macrophages or the smooth muscle cells from stable and unstable plaques. The activity of iNOS in vivo was supported by the immunolocalization of nitrotyrosines, a marker of peroxynitrite production. Nitrotyrosines were detected mostly in macrophages, and in some cases in the extracellular milieu or on the plasma membrane of smooth muscle cells. Nitrotyrosine residues were found in 11/14 (78%) iNOS-positive patients and in only 1/10 (10%) iNOS-negative cases (P<0.01).

2.4. Correlation of immunolocalizations with clinical presentation

To better define the relation between iNOS expression and clinical instability, we separated the patients with stress-induced ischemia (i.e., the Braunwald class I) from those with pain at rest (regrouped in Braunwald classes II and III), and we correlated the immunological findings with the presence of thrombus at morphological examination. As shown on Fig. 5, the expression of iNOS was found in 82% of patients presenting with pain at rest (without significant differences between class II versus class III), whereas it was detected in only 36% of patients from the Braunwald class I who present with a worsening pattern of stress-induced ischemia (P<0.01). The prevalence of thrombus among patients with or without induction of iNOS is also presented in Fig. 5. A thrombus was detected in 66% of cases positive for iNOS and only 18% of iNOS-negative plaques (P<0.01).

Fig. 1. Histopathological characteristics of patients with stable and unstable angina. Plaques from patients with unstable angina were characterized by less fibrosis (panel A), higher cellularity (panel B) and a higher prevalence of thrombus (panel C). * P<0.01 versus patients with stable angina.
Fig. 2. Examples of immunolocalization of macrophages (panel A), iNOS (panel B) TNFα (panel C) and smooth muscle cells (panel D). iNOS was detected in macrophages (arrows on panel B) and in the extracellular space (asterisk on panel B). TNFα was detected in macrophages (arrows on panel C) and in the extracellular space (asterisk on panel C), but not systematically in smooth muscle cells. The extracellular localization of iNOS and TNFα may reflect macrophage necrosis. Original magnification ×40.

3. Discussion

Many experimental data have shown that expression of inducible enzymes may favour plaque instability, but few data are available from living patients with coronary artery disease. In this study, we investigated whether a relation may be found between the enzymatic induction of iNOS and coronary plaque instability in patients with acute coronary syndromes. Mainly, we show that iNOS is indeed expressed in human coronary atherosclerotic lesions from patients with unstable angina, and its presence is associated both with a higher severity of instability and thrombus
formation. In agreement with previous reports [23,24], we detected iNOS expression in the macrophage population. However, iNOS expression can not be regarded simply as a surrogate of macrophage infiltration, because TNFα-positive macrophages were found in both stable and unstable plaques, whereas iNOS expression was only observed in unstable patients.

In pathophysiological conditions, such as heart failure or myocarditis, induction and activation of iNOS may be toxic by production of ‘too much of a good thing’ [33,34]. In the atherosclerotic plaque, however, the role of iNOS remains controversial for the following reasons. On one hand, some deleterious effects are attributed to iNOS, due to the production of peroxynitrite from NO and superoxide [11–14]. On the other hand, it is argued that iNOS activation may be protective, as NO inhibits smooth muscle cell proliferation and leukocyte adhesion [21,22]. As recently reviewed [7], such controversy may result from a dose-dependent difference in the various properties of NO. An overall protective effect (vasorelaxation and...

Fig. 3. Distribution of macrophages, TNFα and iNOS in patients with either stable or unstable angina. * $P<0.01$ versus patients with stable angina.

Fig. 4. Serial sections of an atherectomy sample showing the absence of iNOS expression (panel B) in smooth muscle cells (panel A). Original magnification $\times 40$.

Fig. 5. Prevalence of pain at rest and thrombus among patients expressing (iNOS +) or not expressing (iNOS, −) iNOS. * $P<0.01$ versus iNOS (−) groups.
cytoprotection) results from the production of a ‘basal’ rate of NO (in the picomolar range), whereas cytotoxicity (hyperreactivity, platelet adhesion and protein fragmentation) results from the production of higher rates (nanomolar to micromolar range) of NO and peroxynitrite. An imbalance may happen in atherosclerotic plaque, as a consequence of the downregulation of EcNOS and the induction of both iNOS and neuronal NOS [24]. Another cause of imbalance of NO production in advanced lesions is the decreased expression of superoxide dismutase, an enzyme which prevents peroxynitrite formation [35].

The present study also shows that iNOS expression within the plaque is associated with increased presence of thrombus and more frequent pain at rest. The QCA measurements further demonstrate that the severity of the coronary narrowing was identical between stable and unstable patients (Table 2), and therefore support the concept that dynamic factors (such as thrombogenesis and/or vasoreactivity) can be held responsible for the greater instability.

Two putative mechanisms may relate iNOS to increased vasoreactivity and thrombogenesis. On one hand, peroxynitrite impairs the action of endothelial-released NO [18], thus impairing normal vasoreactivity. On the other hand, peroxynitrite together with superoxide stimulate platelet adhesion and aggregation [13,14]. These two mechanisms – altered vasoreactivity and thrombogenesis – are well known to be operative in unstable plaque, and iNOS expression was strongly correlated with both pain at rest and thrombus. The prevalence of thrombus in unstable plaque is proportional to the score of instability [31] and, as shown here, the same applies to iNOS. Our population of unstable patients seems well representative, as the proportion of thrombus found in the present study is very close from the values reported in both angiography studies [36] and atherectomy studies [31,32]. The risk of blood coagulation is further increased by the production of tissue factor [37], and intracoronary thrombus formation per se will favour focal constriction [38,39].

For technical reasons, we can not directly measure NO release from atherosclerotic plaque in patients. However, the plasma concentration of L-arginine and oxygen (the two substrates of NOS) are higher by far than the apparent $K_m$ of iNOS for these substrates [6,40], indicating that the enzyme should be active in vivo. This is indirectly confirmed by the detection of nitrotyrosine residues in iNOS-positive plaque fragments, reflecting the synthesis of peroxynitrites [20]. Nitrotyrosines were indeed detected in most iNOS-positive fragments. Also, the morphological analysis of DCA fragments only provides small biopsies of the plaque, and atherosclerosis is, by nature, heterogeneous [2]. Because the number of cells and the sample composition can not be identical in all cases, sampling error can not be totally excluded. This may explain why the prevalence of macrophage clusters was higher in the present study than in previous reports [31,32]. In an attempt to limit these errors, we have included in this study only cases in which two or more samples were retrieved.

4. Conclusions

Expression of iNOS may be induced in coronary atherosclerotic plaque from patients with unstable angina. Such induction is selectively found in macrophage clusters, but can not be regarded as a surrogate of macrophage infiltration. A high rate of NO production, which is supported by the detection of nitrotyrosine residues, is associated with a higher prevalence of pain at rest and thrombus detection in the plaque.

Acknowledgements

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