In vitro and in vivo evidence for the role of elastase shedding of CD163 in human atherothrombosis

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Aims
CD163 is a macrophage receptor for haemoglobin–haptoglobin (Hb–Hp) complexes, responsible for the clearance of haemoglobin. We hypothesized that production of soluble CD163 (sCD163) may be due to proleolytic shedding of membrane CD163 by neutrophil elastase, reported to be increased in culprit atherosclerotic plaques. We analysed the relationship between CD163 solubilization and elastase in vitro, in macrophage culture, ex vivo in human atherosclerotic plaque samples, and in vivo, in plasma of patients with coronary artery disease.

Methods and results
Neutrophil elastase was shown to enhance CD163 shedding and to decrease the uptake of Hb–Hp complexes by cultured macrophages. In addition, cultured carotid endarterectomy samples showing features of intraplaque haemorrhage released more sCD163 and elastase/α1-antitrypsin (α1-AT) complexes than non-haemorrhagic plaques (n = 44). Plasma levels of sCD163 and neutrophil elastase (complexed with α1-AT) were measured in patients with an acute coronary syndrome (ACS, n = 42), stable angina pectoris (SAP, n = 28), or normal coronary angiograms without subclinical atherosclerosis (n = 21). Acute coronary syndrome patients had higher sCD163 and elastase/α1-AT complexes plasma concentrations than subjects without coronary atherosclerosis. Circulating sCD163 and elastase/α1-AT complexes were positively correlated in patients with ACS (r = 0.56, P < 0.0002) and SAP (r = 0.62, P < 0.0005).

Conclusion
Our results suggest that neutrophil elastase promotes CD163 shedding, resulting in a decreased clearance of Hb by macrophages, which may favour plaque destabilization. This may be reflected by increased plasma levels of sCD163 and elastase/α1-AT complexes which are positively correlated in patients with coronary artery disease.

Keywords
Atherosclerosis • Intraplaque haemorrhage • CD163 • Elastase • Coronary heart disease • Haemoglobin

Introduction
Intraplaque haemorrhage accelerates the development and the destabilization of atherosclerotic lesions.1–6 A recent study reported that the presence of plaque haemorrhage and increased intraplaque vessel density was associated with the occurrence of future vascular events, whereas macrophage infiltration, large lipid core, calcifications, collagen accumulation, and smooth muscle cell infiltration were not related to clinical outcome.6

Haemolysis of extravasated red blood cells releases haemoglobin (Hb) and leads to its accumulation. Haemoglobin is a strong oxidant and has potent pro-inflammatory properties.5 To restrain these activities, cell-free Hb is captured by haptoglobin (Hp) and subsequently cleared via CD163, a membrane protein expressed by circulating monocytes and tissue macrophages.7 CD163 has also been found in a soluble form (sCD163), in human plasma, and in cell culture supernatants8 after proteolytic cleavage under oxidant and pro-inflammatory conditions.9–11

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sCD163 plasma levels are elevated in patients with coronary artery disease (CAD) and diabetes. Recently, sCD163 plasma levels were reported to be positively correlated with carotid artery intima/media thickness in asymptomatic subjects. Furthermore, sCD163 concentration was suggested to be a predictor of CAD burden. In coronary atherectomy specimens, CD163 expressing cells were positively correlated with the presence of red blood cells and a tissue marker of oxidation. More importantly, CD163 expression was enhanced in unstable vs. stable plaques.

Haemoglobin clearance is markedly reduced by elastase
Since a major function of CD163 on macrophages is to take up Hb–Hp complexes, we reasoned that elastase-induced CD163 shedding may impair this cardinal feature of macrophages. THP-1 cells expressing CD163 were exposed to fluorescence-labelled Hb–Hp complexes to quantify the uptake of these complexes by flow cytometry (Figure 2). As shown in Figure 2, addition of elastase and PMN-conditioned medium induced a dose-dependent decrease of Hb–Hp uptake, an effect inhibited by addition of α1-AT (an inhibitor of elastase). These results were confirmed by confocal microscopy showing a decreased intracellular accumulation of Hb–Hp complexes within cells treated by elastase relative to control cells (Figure 2C).

Elastase cleaves membrane CD163 on monocytes cells
In order to analyse whether neutrophil-derived proteases, and in particular elastase, can cleave membrane-bound CD163, human acute monocytic leukemia cell line (THP-1) cells were differentiated into macrophages using phorbol myristate acetate for 48 h and then treated for 24 h with dexamethasone to induce maximal CD163 expression. Equal numbers of macrophages were then exposed to either purified elastase or medium conditioned by N-formyl-methionine-leucine-phenylalanine-stimulated polymorphonuclear neutrophils (PMNs). CD163 expression was analysed on the membrane fraction of macrophages by western blot (Figure 1A) and by flow cytometry (Figure 1B and C). Purified elastase, but neither cathepsin G nor plasmin, decreased membrane-bound CD163 in a dose-dependent manner (Figure 1A and B). Similar results were obtained when incubation was performed with activated PMN-conditioned medium (Figure 1C). This decreased presence of membrane-bound CD163 in macrophages was in part inhibited by α1-AT (the natural inhibitor of elastase).

Interestingly, a negative correlation was observed between membrane CD163 assessed by flow cytometry and sCD163 quantified by ELISA in the supernatant (r = −0.692, P < 0.0001, n = 23). A Supplementary material online, Figure S1). Elastase and PMN-conditioned medium increased CD163 release in a time- and dose-dependent manner, as confirmed by ELISA (Figure 1D–G), confirming a direct effect of elastase on CD163 shedding.

Methods
See Supplementary material online, Supplemental methods.

Results

Elastase shedding of CD163 in human atherothrombosis

Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1E

Figure 1F

Figure 1G
Elastase/α1-AT complexes and sCD163 levels were assessed by ELISA in the conditioned medium from CPs, with or without intra-plaque haemorrhage, and from paired NCPs. As expected, CPs released more elastase/α1-AT complexes and sCD163 than corresponding adjacent areas (P, 0.001). Elastase/α1-AT levels were increased in conditioned medium from haemorrhagic relative to non-haemorrhagic plaques (CP-H vs. CP-NH, P = 0.004 and NCP-H vs. NCP-NH, P = 0.018) (Figure 3C). sCD163 was found predominantly in conditioned medium of haemorrhagic CPs compared with non-haemorrhagic ones, as determined by ELISA and western blot (Figure 3D–E). Of note, CPs released more proteins than NCPs due to their pathological state (normalization to tissue wet weight was used). Interestingly, positive associations were observed between the amount of haemoglobin and concentrations of elastase/α1-AT complexes (r = 0.714; P < 0.001) and sCD163 (r = 0.795; P < 0.001) in the conditioned medium. It is noteworthy

**Figure 1** Elastase cleaves membrane CD163 on monocytic cells. Western blot analysis showing CD163-membrane expression in macrophages after treatment with elastase, cathepsin G (CG) and plasmin (Pn) for 1 h (A). Macrophages were then incubated with elastase or polymorphonuclear neutrophil-conditioned media (PMN-CMs) (0.25–0.5 mL/mL) in the presence or absence of α1-antitrypsin (α1-AT). CD163 expression was then quantified by flow cytometry (B and C) and the release of soluble CD163 was determined by ELISA in cell supernatants (D–G). Results are expressed as means ± SD of three independent experiments. *P < 0.05 when compared with non-treated cells. #P < 0.05 when compared with non-α1-antitrypsin-treated cells.
that sCD163 was positively associated with elastase/α1-AT complexes \((r = 0.676; \ P < 0.001)\). These data suggest that neutrophil-associated elastase present in haemorrhagic plaques could interact with CD163 from macrophages and potentially promote its shedding.

**Elastase triggers CD163-sheding ex vivo**

In order to determine whether elastase mediates CD163 shedding, we performed ex vivo experiments on human carotid endarterectomy samples. Culprit plaque and NCP were divided into two similar segments, each one was incubated with or without exogenous elastase (10 nM, \(n = 15\)) or with elastase inhibitor, α1-AT (200 nM, \(n = 15\)). The sCD163 levels generated during the 24 h tissue-incubation period were then assessed in the conditioned medium. After addition of exogenous elastase, significantly more sCD163 was released by CP than by NCP \((P < 0.05,\) not shown). Conversely, addition of α1-AT (an inhibitor of elastase) led to a decrease in CD163 shedding in CP \((P < 0.05)\), but not in NCP. When the presence or absence of intraplaque haemorrhage was taken into account, addition of elastase at the time of incubation significantly increased CD163 released into the conditioned medium of haemorrhagic plaques relative to non-haemorrhagic ones (Figure 4A). Addition of α1-AT decreased the shedding of sCD163 more markedly in haemorrhagic plaques (Figure 4B). Western blot analysis for detection of sCD163 showed a more intense degradation pattern of CD163 when CP were incubated in the presence of elastase for 24 h (Figure 4C). Interestingly, similar but more intense bands were observed when exogenous elastase was added, suggesting that endogenous elastase was sufficient to produce CD163 proteolytic fragments. This effect was prevented by incubation with α1-AT.

**CD163 and elastase co-localize in human atherothrombotic carotid plaques**

To investigate the possible in vivo interaction between CD163 and elastase, immunohistofluorescence staining was performed in...
human haemorrhagic and non-haemorrhagic carotid plaques (Figure 5, Supplementary material online, Figure S3). As previously reported,\textsuperscript{15,24} we observed a strong CD163 immunostaining in haemoglobin-rich areas. However, CD163 was also expressed in plaques without macroscopical or biochemical detectable levels of haemoglobin in their conditioned medium (Figure 5, right panels). In these plaques, immuno-fluorescence allowed detection of small amounts of haemoglobin (probably residual from an old/
small haemorrhage), but no elastase could be detected (data not shown), in contrast to haemorrhagic plaques, which contained not only haemoglobin but also blood-derived cells, including neutrophils expressing elastase (Figure 5, left panels). Double immunostaining for haemoglobin/elastase and haemoglobin/CD163 showed that some cells that have engulfed large amounts of haemoglobin expressed CD163 (but not all of them). Elastase and CD163 immunodetection on consecutive sections suggests that the interaction elastase-CD163 is possible in vivo, since they were localized in the same area. Elastase-positive immunostaining was always associated with recent intraplaque haemorrhage as attested by the presence of intact erythrocytes (Figure 5C, inset). In contrast to a previously published study, neither endothelial cells (neo-vessels, Nv are indicated in Figure 5) nor macrophages were positive for elastase immunostaining. In haemorrhagic plaques, CD163 immunostaining was less intense than in plaques...
containing traces of an old/small haemorrhage (Figure 5, right panels) probably due to limited protease activity (and thus little or no CD163 shedding).

**sCD163 and elastase/α1-AT complexes are coordinately elevated and positively associated in plasma of patients with coronary artery disease**

Characteristics of the BIOCORE-1 study population are presented in Table 1. Body mass index, gender, and distribution of principal risk factors were similar between groups. Patients with CAD [acute coronary syndrome (ACS) and stable angina pectoris (SAP) groups] were older, were more often on statins (resulting in lower LDL cholesterol levels), and had lower HDL cholesterol levels than patients without atherosclerosis.

Acute coronary syndrome and SAP patients had higher sCD163 plasma levels than patients without atherosclerosis (593.2 ± 264.6, 543.4 ± 205.9, and 396.8 ± 159.6 ng/mL, respectively; ANOVA, P = 0.01) (Table 2). A non-significant trend towards increased plasma levels of elastase/α1-AT was observed in ACS and SAP patients relative to patients without atherosclerosis (Table 2). A positive correlation between plasma levels of sCD163 and elastase/α1-AT complexes was observed in the overall population.
Table 1  Study population

<table>
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<tr>
<th>Risk factors at baseline</th>
<th>ACS (n = 42)</th>
<th>SAP (n = 28)</th>
<th>NA (n = 21)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, mean (SD)</td>
<td>58.8 (12.8)</td>
<td>66.9 (6.9)</td>
<td>53.4 (10.4)</td>
<td>&lt;0.001</td>
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<tr>
<td>Male sex, n (%)</td>
<td>34 (81)</td>
<td>24 (85.7)</td>
<td>18 (85.7)</td>
<td>0.88</td>
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<tr>
<td>BMI, kg/m², mean (SD)</td>
<td>27.5 (4.2)</td>
<td>27.3 (4.2)</td>
<td>27.3 (5.1)</td>
<td>0.95</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>17 (40.5)</td>
<td>15 (53.6)</td>
<td>9 (42.9)</td>
<td>0.54</td>
</tr>
<tr>
<td>SBP, mmHg, mean (SD)</td>
<td>142.4 (22.3)</td>
<td>140.4 (23)</td>
<td>130.6 (19.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>DBP, mmHg, mean (SD)</td>
<td>77.7 (13.3)</td>
<td>74.4 (11.9)</td>
<td>75 (12.9)</td>
<td>0.46</td>
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<tr>
<td>Diabetes mellitus, n (%)</td>
<td>14 (33.3)</td>
<td>11 (39.3)</td>
<td>6 (28.6)</td>
<td>0.73</td>
</tr>
<tr>
<td>Current smokers, n (%)</td>
<td>22 (52.4)</td>
<td>10 (35.7)</td>
<td>9 (42.9)</td>
<td>0.38</td>
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</tbody>
</table>

Table 2  Plasma concentration of sCD163 and α1-AT/HLE

<table>
<thead>
<tr>
<th></th>
<th>ACS (n = 42)</th>
<th>SAP (n = 28)</th>
<th>NA (n = 21)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCD163 (ng/mL)</td>
<td>593.2 (264.6)*</td>
<td>543.4 (205.9)**</td>
<td>396.8 (159.6)</td>
<td>0.01</td>
</tr>
<tr>
<td>α1-AT/HLE (ng/mL)</td>
<td>51.8 (40.5)</td>
<td>44.6 (27.4)</td>
<td>33 (17)</td>
<td>0.146</td>
</tr>
</tbody>
</table>

Discussion

This is the first study to demonstrate that elastase cleaves membrane CD163 on mononuclear cells and that haemoglobin clearance is drastically reduced by elastase activity in vitro. We also show that the release of sCD163 is increased in haemorrhagic relative to non-haemorrhagic plaques and is associated with a higher plaque content of elastase conveyed by blood leucocytes. By immunohistochemistry, we report that the interaction between elastase and CD163 is possible in vivo, since they were localized in the same area within the plaque.

Finally, we show a positive correlation between sCD163 and elastase/α1-AT complexes both in plasma of subjects with CAD and in medium conditioned by human carotid atherothrombotic plaques. These findings suggest a potentially important role of elastase shedding of CD163 in triggering coronary plaque instability in humans via intraplaque accumulation of haemoglobin.

CD163 solubilization by elastase reflects neutrophil activation

Our results demonstrate in vitro using a monocytic cell line differentiated into macrophages expressing CD163, and ex vivo, in human carotid specimens that neutrophil elastase promotes CD163 shedding. Elastase has been detected in atherothrombotic plaques, but not in normal human arteries. It has been

[r = 0.57 (95% CI: 0.41–0.70), P < 0.001]. Interestingly, this correlation remained statistically significant in ACS or SAP patients [ACS: r = 0.56 (95% CI: 0.30–0.75), P < 0.0002; and SAP: r = 0.62 (95% CI: 0.31–0.80), P < 0.0005, Figure 6A and 8] but not in patients without atherosclerosis (r = 0.06 (95% CI: −0.43–0.53), P = 0.81). Finally, we tested for a possible trend for α1-AT complex levels to decrease across groups with increasing severity (controls, stable angina, and ACS). However, no linear or quadratic trend was identified (P = 0.0515 and P = 0.78, respectively).

ACS, acute coronary syndrome; SAP, stable angina pectoris; NA, no atherosclerosis; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; EKG, electrocardiogram.

Results are expressed as mean; standard deviation is indicated in parentheses.

aGlobal comparison assessed by ANOVA. Non-parametric Kruskal–Wallis test was used for comparisons of sCD163 levels: ACS vs. NA.

bP = 0.02 (ACS vs NA) after adjustment by Hochberg’s method for multiple comparisons.

cP = 0.02 (SAP vs NA), after adjustment by Hochberg’s method for multiple comparisons.
proposed that neutrophils, conveyed by the blood into the plaque or trapped within the intraplaque hematoma, could be a source of elastase. In our study, elastase and CD163 were localized in the same areas of haemorrhagic plaques, suggesting that the interaction elastase-CD163 is possible in vivo. Assessment of circulating sCD163 could therefore reflect both the presence of macrophages specialized in haemoglobin clearance and elastase activity associated with activated neutrophils. Evidence of neutrophil activation and degranulation in patients with ACS has been reported and neutrophil infiltration was observed in ruptured, thrombosed coronary plaques in patients. Even though the principal source of extracellular elastase in haemorrhagic plaques is likely to be activated neutrophils, it should be recognized that other cell types have been reported to produce elastase as well. In our experimental conditions, neither CD163-positive cells nor endothelial cells were shown to contain elastase (immunohistology). None of the haemorrhagic carotid samples used in our study displayed ruptured plaques that could present an intraluminal thrombus (Supplementary material online, Figure S3 shows intraplaque haemorrhage and an alpha-actin positive fibrous cap corresponding to the carotid section presented in Figure 5). However, it cannot be excluded that in some cases in vivo, a luminal thrombus may account for elastase release into the plasma.

It has been suggested that the plasma leucocyte elastase concentration may be a sensitive diagnostic marker of CAD and that high elastase concentrations may indicate the presence of culprit atherosomatous plaques. In plasma, elastase is immediately inhibited by forming a complex with the abundant α1-AT. Here, we measured elastase levels in plasma by an antigenic method (ELISA) able to quantify elastase bound to α1-AT. In view of the high concentration of plasma α1-AT, it is likely that shedding of CD163 takes place within atherosclerotic plaques. Indeed, activated neutrophils persistently express active elastase on their cell surface, which is remarkably resistant to inhibition by naturally occurring proteinase inhibitors, particularly at sites of inflammation. These observations could explain the increased level of sCD163 obtained in the medium conditioned by haemorrhagic human atherosclerotic carotid plaques. These plaques also presented the highest α1-AT/elastase complex levels when compared with non-haemorrhagic ones. This is in agreement with our previous observations showing that the levels and activity of elastase in the conditioned medium of CPs with intraplaque haemorrhage are increased compared with those from non-complicated plaques or healthy tissue. We also have shown ex vivo, using human carotid samples, that CD163 is released in higher amounts when exogenous purified elastase was added at the time of incubation. In contrast, CD163 release was markedly decreased by addition of α1-AT, suggesting that the endogenous elastase, present in CPs, is sufficient to shed CD163 in this atherothrombotic environment. Although limited effect on CD163 shedding was observed for cathepsin G or plasmin, we cannot exclude the contribution of other proteases in CD163 release, such as MMP12 and other macrophage sheddases. Also, other stimuli potentially relevant in atherosclerotic plaques such as oxidative stress may participate in CD163 solubilization. Here, we report that CD163-expressing cells are found in both non-haemorrhagic and haemorrhagic plaques and that addition of elastase promoted its shedding. This suggests that the presence of soluble CD163 reflects the presence of CD163-macrophage burden associated with elastase activity, potentially conveyed by intraplaque haemorrhage. This biomarker of intraplaque haemorrhage is of major importance since this feature of plaque composition is an independent predictor of occurrence of future vascular events.

### Functional consequences of CD163 shedding

The primary mechanism protecting against the deleterious effects of free Hb is governed by a cooperation between the plasma protein Hp and the scavenging capacity of CD163. Haptoglobin binds to free-Hb forming a Hb–Hp complex which has been reported to be exclusively cleared by CD163. In agreement with previous reports in which CD163 expression was shown in

![Figure 6](https://academic.oup.com/eurheartj/article-abstract/33/2/252/435951/7/6)
coronary and aortic lesions, we have observed that CD163 is expressed in human carotid atherosclerotic plaques, principally, but not only, in those with intraplaque haemorrhage. A recent study reports the presence of a novel macrophage phenotype in haemorrhaged atherosclerotic plaques characterized by high levels of CD163, an increased capacity for Hb clearance and for cell survival associated with less hydrogen peroxide release and reduced oxidant stress. CD163-expressing cells have been shown to be positively correlated with 4-hydroxy-2-nonenal (an end-product of lipid peroxidation) and with glycoprotein A (specific to erythrocytes). It thus seems reasonable to speculate that the release of Hb due to intraplaque haemorrhage directs monocyte/macrophage differentiation towards an antioxidant and atheroprotective phenotype by stimulation of CD163 expression, therefore promoting Hp/Hb clearance.

Cleavage of CD163 by elastase has a functional consequence since it results in the decreased clearance of Hp–Hb complexes by macrophages. Extracellular haemoglobin accumulation in intraplaque haemorrhage may favour plaque vulnerability by promoting oxidation and associated deleterious effects.

Circulating CD163 in atherosclerosis

CD163 has been reported to exist in a soluble form in human plasma under pathological conditions associated with macrophage proliferation and activation such as rheumatoid arthritis, haemophagocytosis, sepsis, myelomonocytic leukemia, and diabetes. We show here that sCD163 levels were significantly increased in plasma of patients from the BIOCORE-1 study who presented with an ACS or with SAP relative to subjects without detectable atherosclerosis. Previous studies demonstrated that sCD163 concentration is a predictor of CAD extent, independently of conventional risk factors such as age, hyperlipidaemia, hypertension, and smoking status. Moreover, circulating sCD163 is a biomarker of subclinical atherosclerosis since its plasma concentration correlates with carotid artery intima/media thickness in asymptomatic patients, and this association remains significant after adjustment for traditional cardiovascular risk factors and other inflammatory biomarkers. Of note, in BIOCORE-1, plasma from patients were obtained 1 month after their index hospitalization; this indicates that sCD163 is a

**Figure 7** In haemorrhagic plaques, the presence of activated neutrophils may lead to proteolysis of membrane CD163 by elastase and subsequent accumulation of pro-oxidant haemoglobin. Impairment of haemoglobin clearance due to increased blood-borne protease activity, associated with intraplaque haemorrhage, could favour plaque vulnerability. In plasma of atherothrombotic patients, increased levels of the shed form of CD163 (sCD163) could reflect an overwhelmed anti-protease status combined with deficient haemoglobin clearance and thus represent an index of plaque vulnerability.
persistent marker of atherothrombosis and its increased plasma concentration in CAD patients is neither due to acute coronary events nor to percutaneous coronary intervention. Measurement of sCD163 in larger cohorts of patients will be required to further assess the diagnostic value of circulating sCD163 for plaque instability. More importantly, we show a positive correlation between sCD163 and elastase/α1-AT complexes in the plasma of patients from the BIOCORE-1 study (r = 0.57, P < 1.0e–07), which suggests that leucocyte elastase mediates, at least in part, CD163 shedding.

In conclusion, our results, summarized in the Figure 7, suggest in the one hand, that shedding of CD163 by elastase conveyed by intraplaque haemorrhage may play, via decreased Hb clearance by macrophages, a deleterious role in human atherothrombotic plaque instability and therefore in triggering acute coronary events. Leucocyte elastase could thus represent a novel therapeutic target. On the other hand, increased plasma levels of sCD163 in patients with CAD may be a testimony of this process and could be used to further assess patient prognosis.

Supplementary material

Supplementary material is available at European Heart Journal online.

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Conflict of interest:

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Supplementary material

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associated with increased levels of proteolytic markers of neutrophil activation. Atherosclerosis 2007;194:334–341.


