European Heart Journal (2005) **26**, 2200–2205
doi:10.1093/eurheartj/ehi461

EUROPEAN SOCIETY OF CARDIOLOGY®

Preclinical research

In vivo temperature heterogeneity is associated with plaque regions of increased MMP-9 activity

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Received 29 June 2004; revised 24 May 2005; accepted 22 July 2005; online publish-ahead-of-print 5 September 2005

KEYWORDS
Macrophages;
Intravascular thermography;
Inflammation;
Metalloproteinases

Aims Plaque rupture has been associated with a high matrix metalloproteinase (MMP) activity. Recently, regional temperature variations have been observed in atherosclerotic plaques *in vivo* and ascribed to the presence of macrophages. As macrophages are a major source of MMPs, we examined whether regional temperature changes are related to local MMP activity and macrophage accumulation.
Methods and results Plaques were experimentally induced in rabbit ($n = 11$) aortas, and at the day of sacrifice, a pull-back was performed with a thermography catheter. Hot ($n = 10$), cold ($n = 10$), and reference ($n = 11$) regions were dissected and analysed for smooth muscle cell (SMC), lipids (L), collagen (COL), and macrophage (MΦ) cell densities (%); a vulnerability index (VI) was calculated as $VI = M\Phi + L/(SMC + COL)$. In addition, accumulation and activity of MMP-2 and MMP-9 were determined with zymography. Ten hot regions were identified with an average temperature of $0.40 \pm 0.03°C$ ($P < 0.05$ vs. reference) and 10 cold regions with $0.07 \pm 0.03°C$ ($P < 0.05$ vs. hot). In the hot regions, a higher macrophage density (173%), less SMC density (77%), and a higher VI (100%) were identified. In addition, MMP-9 (673%) activity was increased. A detailed regression analysis revealed that MMP-9 predicted hot regions better than macrophage accumulation alone.
Conclusion *In vivo* temperature measurements enable to detect plaques that contain more macrophages, less SMCs, and a higher MMP-9 activity.

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Introduction

Rupture of atherosclerotic plaques with subsequent thrombus formation is the most important mechanism underlying acute coronary syndromes.[1] These disrupted atherosclerotic plaques typically consist of a large lipid pool covered with a thin fibrous cap with large infiltrates of inflammatory cells in the shoulder of these plaques.[2] The rupture is believed to result from the existence of local weak spots either within the cap covering the lipid core or within the shoulder of these plaques.[3] These weak spots have been associated with increased matrix metalloproteinase (MMP)[4,5] activity and subsequent increased breakdown of extracellular matrix including collagen.[1]

Recently, temperature differences have been measured in explanted human plaques and correlated positively with cell densities.[6] Several groups, including our group, confirmed the existence of temperature differences *in vivo* and reported that they were associated with symptoms of unstable angina, previous myocardial infarction,[7,8] or the presence of macrophages in experimentally induced plaques.[9,10]

As macrophages are an important source of MMPs in atherosclerotic plaques and as rupture of plaque has been associated with increased activity of MMPs,[3,11] we evaluated the hypothesis that temperature heterogeneity within the plaque is associated with both macrophage accumulation and increased MMP activity. To that end, regional temperature heterogeneity was measured under well-controlled experimental conditions, and the temperature heterogeneity was used to select tissue, which was then tested for macrophage area (%) and MMP activity.

Methods

Instrumentation

New Zealand white male rabbits ($n = 11$; weight $= 3.6 \pm 0.2$ kg) (Harlan, The Netherlands) were fed a high (2%) cholesterol diet, for a period of 2 months. The rabbits were anaesthetized with an

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intramuscular injection of a 2:1 mixture of ketamine and xylazine and the marginal ear artery was canulated for arterial pressure measurement with a fluid-filled catheter (Amatek Inc., PA, USA) and for arterial blood withdrawal. Next, a 40 MHz intravascular ultrasound (IVUS) catheter (CVUS, Boston Scientific, Maastricht, The Netherlands) was advanced through the guiding catheter and located 1 cm upstream of the left renal artery, and a motorized pull-back was performed at a speed of 0.5 mm/s, spanning an arterial segment of 7 cm. Denudation within this pre-defined segment was performed by twisting and pulling back an inflated Fogarty balloon (3Fr, Applied Medical, Laguna Hills, CA, USA) over a length of 5 cm. After an 8-week follow-up period, the right femoral artery was dissected for the introduction of a 5Fr sheath. An IVUS pull-back was repeated through the denuded segment. Subsequently, a pull-back was performed with a temperature catheter (as described subsequently) over the similar arterial segment as described earlier. On the basis of previous findings, showing an increased temperature heterogeneity in reduced flow,[12,13] a standard balloon angioplasty catheter inserted through the carotid artery was inflated proximal to the area of interest to occlude the aorta and impede flow. Following euthanasia of the rabbit (Euthasate®; 3 mL/kg), the abdomen was opened and the arterial segment of interest was dissected and snap frozen in liquid nitrogen.

All experiments were performed in accordance with institutional regulations and the guiding principles for the care and use of laboratory animals published by the US as approved by the Council of the American Physiological Society. The lipid profile was measured according to well-established enzymatic–calorimetric methods (Roche Diagnostics, IN, USA).

IVUS and thermography

The video-taped IVUS images were digitized at intervals of 0.5 mm with a resolution of 800 × 600 pixels and stored in a standard PC. Next, the lumen and the acoustic interface between media and adventitia were traced semi-automatically by a well-validated software package,[14] and the lumen area and media-bounded area were calculated from these contours. Wall area was defined as the difference between these two areas. The thermography catheter (Thermocore Medical Systems Inc.) has been described in detail elsewhere.[9,15] Briefly, the over the wire, catheter consists of four thermistors, touching the vessel wall. Accuracy of each of the thermistors is 0.006°C. The thermography catheter was connected to a dedicated pull-back system, which allowed a constant pull-back speed of 0.2 mm/s. Finally, the average temperature of the reference region, i.e. at the region downstream of the denuded region, was subtracted from each temperature curve for comparison between animals.

Tissue harvesting and histological analysis

A segment of 500 μm with the temperature peak in its centre was harvested for immunohistochemical and zymographic analyses. This 500 μm block was then divided into three blocks: two of 100 μm blocks located at both extremities of the specimen and one central block of 300 μm. The 100 μm blocks were used for zymography, whereas the 300 μm block was used for immunohistochemistry. The reference segment was taken downstream of the denudation area. Immunohistochemistry was performed on 5 μm cross-sections obtained from fresh–frozen tissue. These cross-sections were stained for macrophages (RAM-11; Dako Diagnostics BV, Glostrup, Denmark), smooth muscle cells (SMCs) (α-actin; Dako Diagnostics BV), and MMP-2 (Biogenesis, USA). Areas of macrophages, SMCs, and MMP-2 were calculated relative to the vessel area (Clemex Technologies Inc., Quebec, Canada) and are reported as relative densities (%). Lipid content (%) was measured using Oil-red-O staining, and collagen content was measured using Picrosirius red (%). A vulnerability index (VI) was calculated and was defined as the sum of the amount of macrophages and lipids divided by the sum of SMC content and collagen content.[16] Zymography was performed as previously described.[17]

Data analysis and statistics

To assure appropriate alignment of the temperature measurements with histology, the lower end of the ostium of the left renal artery was used as a reference for both the temperature measurements and the histology. At the end of the experiment, the vessel segment starting from the lower end of the left renal artery with a length of 7 cm was dissected and the amount of shrinkage was monitored. This ratio was multiplied by the location in the temperature curve to obtain the position in the tissue specimen. Hot and cold regions within the denuded area were selected on the basis of the median of the temperature data obtained in the plaque region. Subsequently, differences between hot, cold, and reference regions were tested with analysis of variance. Regression analysis was performed applying linear regression in a standard package. All tests have been performed two-sided. A *P*-value less than 0.05

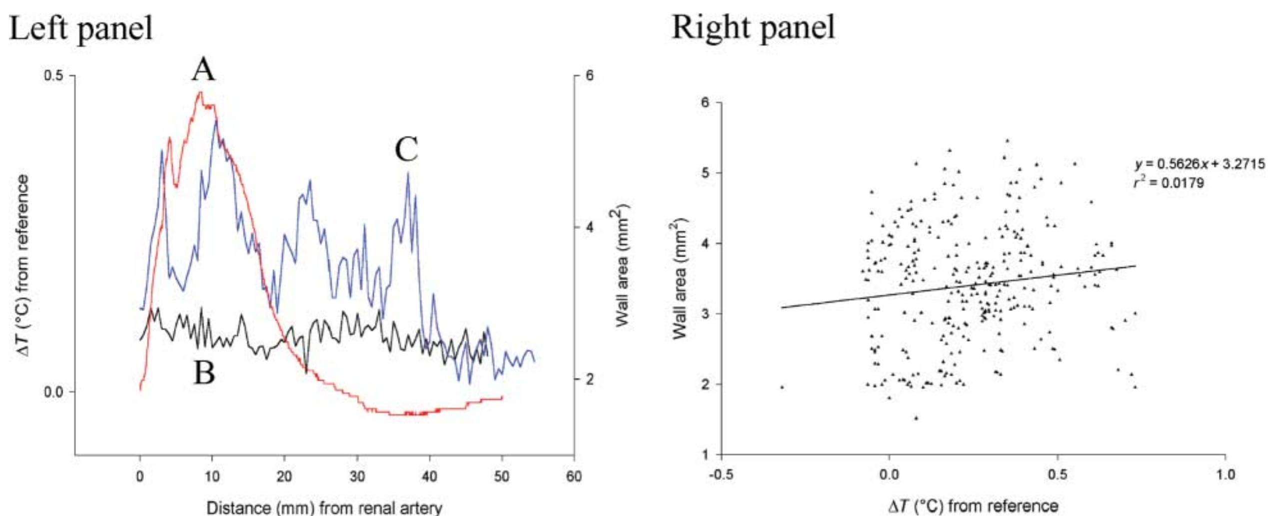

Figure 1 A representative example of an IVUS pull-back and a temperature pull-back for a single rabbit (left panel: A, temperature; B, wall area before injury; C, wall area after injury) and for the entire group of rabbits (right panel). Both situations show an absence of relationship between IVUS-derived wall thickness and temperature heterogeneity.

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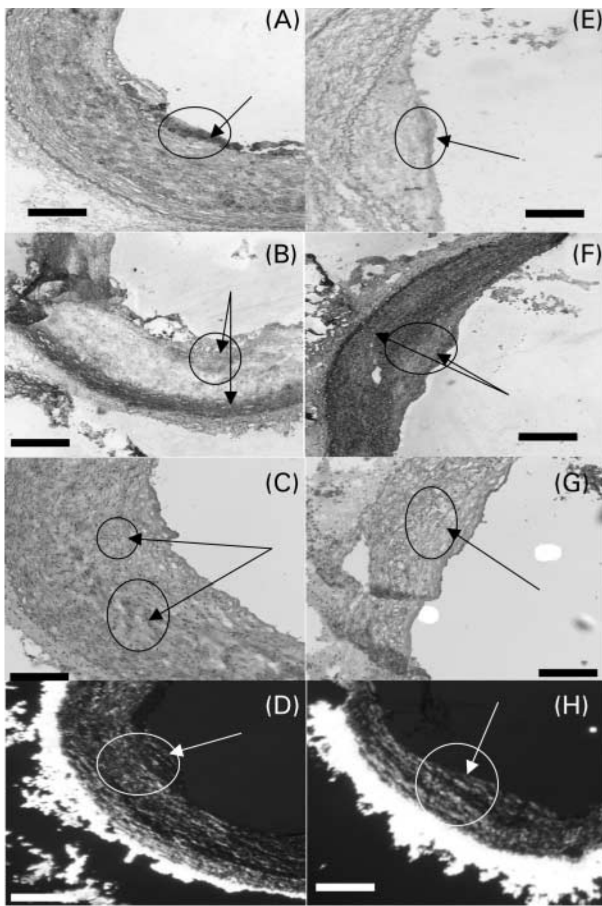

Figure 2 A representative example of the cellular composition of hot (left column) and cold regions (right column). The first row shows the macrophages (RAM-11 stain; *A* and *E*), the second row the SMCs (α-actin stain; *B* and *F*), the third row the lipids (Oil-red-O stain; *C* and *G*), and the last row the collagen (Picrosirius red stain; *D* and *H*). Bar: 500 μm.

was considered statistically significant. All values are reported as mean ± SEM.

Results

Animals and serum lipids

All animals survived the procedure. The cholesterol levels obtained at 8 weeks of diet were 34.5 ± 2.7 mM, which is ~40-fold increase with respect to the values of a normocholesterolaemic control group of rabbits (0.9 ± 0.1 mM; $n = 8$). These rabbits, exhibiting similar characteristics in terms of blood pressure, weight, and sex as the intervention group, have been followed over time for their plasma levels of lipids. No additional experiments were performed.

IVUS and thermography measurements

The temperature (peak value) in the previously denuded areas was 0.4 ± 0.1°C higher than that in the reference segments, whereas the cold region (0.06 ± 0.1°C) displayed a similar temperature as that of the reference region (0°C). The increase in temperature in the denuded area showed a heterogeneous pattern unrelated to the thickness of the plaque (*Figure 1*).

Immunohistochemistry and MMP activity

Identification of tissue on the basis of temperature heterogeneity resulted in the identification of macrophage-rich and SMC-poor regions within the plaque (*Figure 2*). Plaque creation by itself induced more active-MMP-2 accumulation ($P < 0.05$; *Table 1*; *Figures 3* and *4*), whereas selection by temperature identified an increase in MMP-9 ($P = 0.03$; *Table 1*; *Figures 3* and *4*), a higher macrophage density (%) ($P = 0.02$), a higher VI ($P = 0.017$), less SMC ($P = 0.037$; *Table 1*; *Figure 5*), and less collagen ($P = 0.04$; *Table 1*; *Figure 5*).

Table 1 Immunohistochemistry and zymography-derived parameters of the aorta of atherosclerotic rabits ($n = 31$ segments in 11 rabbits)

	Hot region	Cold region	Reference region
Zymography			
Active MMP-2 (OD)	988 ± 213	1015 ± 353	144 ± 42*,†
Pro-MMP-2 (OD)	6624 ± 1031	4285 ± 327	3837 ± 452*
MMP-9 (OD)	1286 ± 412	191 ± 55‡	560 ± 162**
Histology			
Macrophages (%)	10.6 ± 2.7	6.1 ± 1.2‡	0
Smooth muscle cells (%)	18.4 ± 4.9	23.9 ± 0.9‡	0
Collagen (%)	7.2 ± 1.2	12.7 ± 2.9	0
Lipids (%)	7.0 ± 1.7	8.0 ± 2.7	0
MMP-2 (%)	0.8 ± 0.3	0.8 ± 0.3	0
VI-index	1.0 ± 0.3	0.5 ± 0.1‡	0

All values are mean ± standard error of the mean. MMP, matrix metalloproteinase; OD, optical density; VI, vulnerability index as determined from the literature.[15] $^{*}P < 0.05$, hot vs. reference region; $^{\dagger}P < 0.05$, cold vs. reference region; $^{\ddagger}P < 0.05$, hot vs. cold region; $^{**}P = 0.07$, hot vs. reference region for MMP-9, active MMP-2, and pro-MMP-2 are determined by zymography and signify enzyme function, while MMP-2 (%) identifies total protein content. In each rabbit, three segments are analysed: hot, cold, and reference regions. In two rabbits one segment was missing due to technical reasons, leading to 31 segments.

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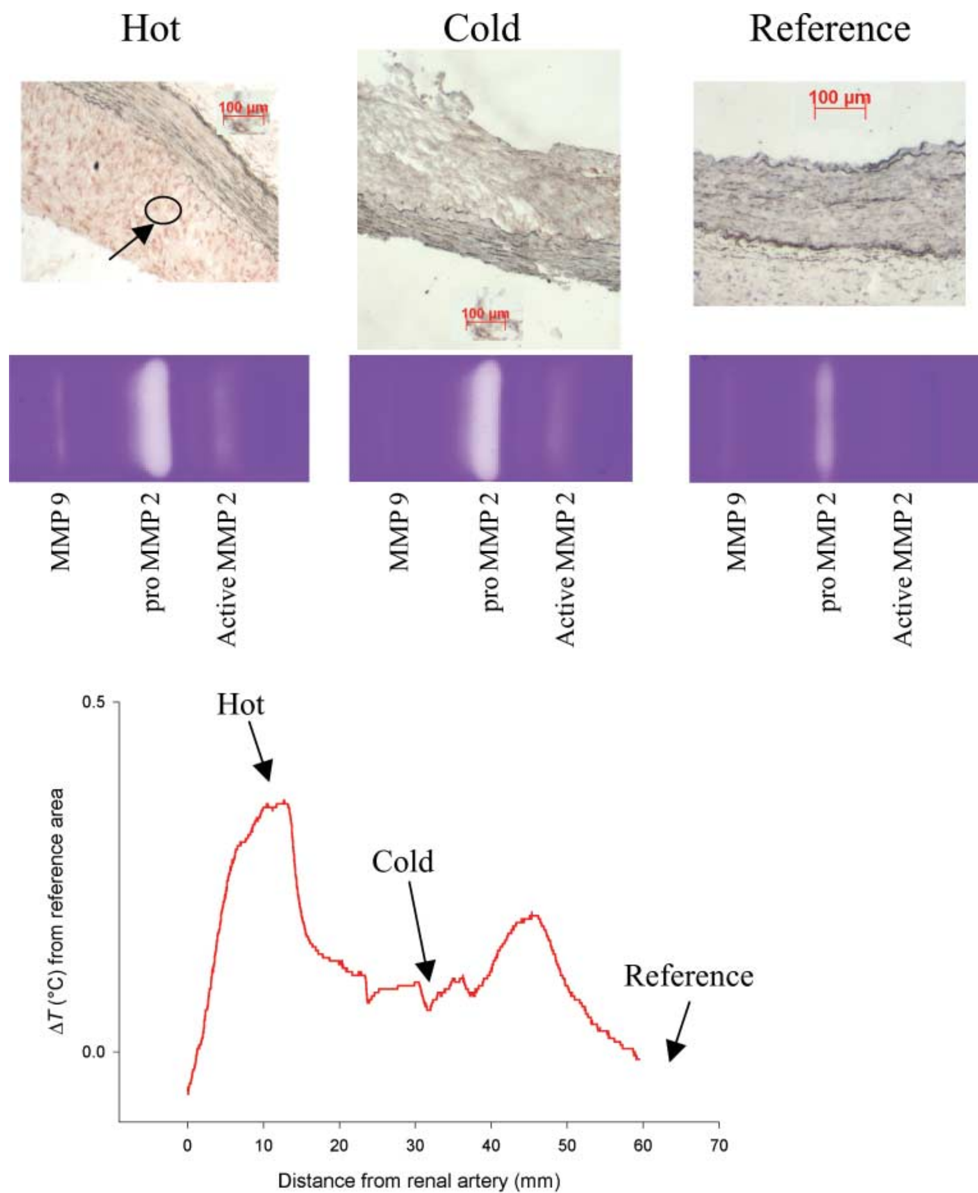

Figure 3 A representative example of hot, cold, and reference regions containing zymography results of pro-MMP-2, MMP-2, and MMP-9 (top) and a temperature curve (bottom) of the same rabbit. In addition, immunohistochemistry of the MMP-2 staining is shown.

Regression analysis revealed that temperature heterogeneity (ΔT) was directly associated with VI ($VI = 3.6 \times \Delta T - 0.32$, $P = 0.003$; $r = 0.8$), but VI was not related with IVUS-derived wall area ($VI = 0.5 \times 10 - {}^{2} \times$ wall area $- 0.9$, $r = 0.002$). Further analysis revealed a direct relationship of macrophages with temperature heterogeneity ($\Delta T = 1.4 \times 10^{-2} \times M\Phi\% + 7.7 \times 10^{-2}$, $P < 0.05$; $r = 0.4$), of pro-MMP-2 with temperature heterogeneity ($\Delta T = 3.0 \times 10^{-5} \times$ pro-MMP-2, $P = 0.05$; $r = 0.4$), and an inverse relationship of SMCs with temperature heterogeneity ($\Delta T = -1.02 \times SMC\% + 0.7$, $P = 0.03$; $r = 0.7$), whereas the relationship between temperature heterogeneity and MMP-9 was positively correlated ($\Delta T = 1.1 \times 10^{-4} \times$ MMP-9 $+ 9.2 \times 10^{-2}$, $P < 0.05$; $r = 0.5$). In contrast, IVUS-derived wall area was not related to.

To investigate whether macrophages or MMPs were more important for explaining the variation in temperature heterogeneity, we performed multi-regression analysis with both MMPs and macrophages as independent factors: ($\Delta T = 8.3 \times 10^{-5} \times M\Phi\% + 8.3 \times 10^{-5} \times$ MMP-9 $+ 5.5 \times 10^{-2}$, $P < 0.05$; $r = 0.6$). Only MMP appeared significant in this equation. Further study with stepwise analysis revealed that the following model appeared optimal ($\Delta T = 1.1 \times 10^{-4} \times$ MMP-9 $+ 9.4 \times 10^{-2}$, $P < 0.05$; $r = 0.5$).

Discussion

Vulnerable plaques have been associated with more macrophages, more lipid accumulation, less collagen accumulation, less SMC accumulation, and a higher MMP activity.[2,4] On the basis of these findings, it has been postulated that accumulation of MMPs, produced either by macrophages or by SMCs, leads to local collagen degradation within the extracellular matrix.[4,11] We found that *in vivo* temperature heterogeneity in atherosclerotic plaques of hypercholesterolaemic rabbit aortas was associated with

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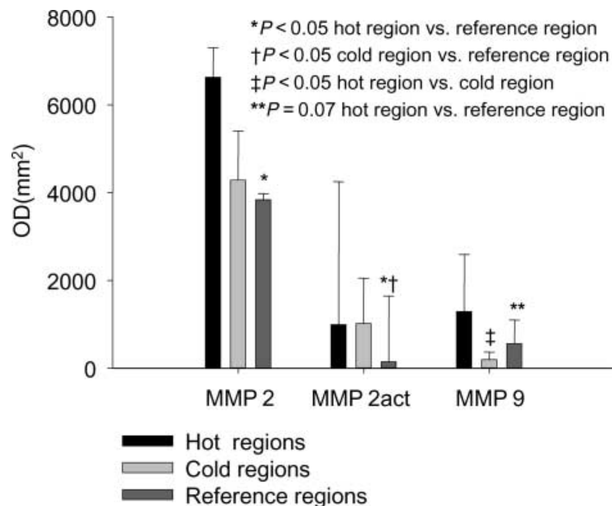

Figure 4 Metalloproteinase activity analysis (MMP-2 and MMP-9) of hot (black bars) plaques, cold (light grey bars) plaques, and the reference region (dark grey bars) of the aorta of atherosclerotic rabbits ($n = 11$).

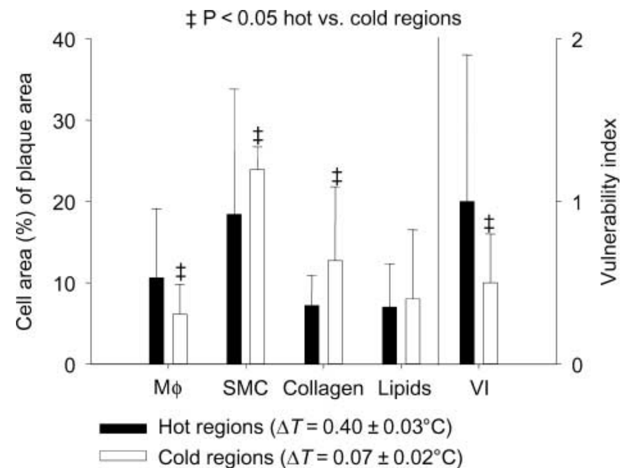

Figure 5 Cellular components of hot (filled squares) plaques and cold (open squares) plaques in the infrarenal aorta of a series of 11 atherosclerotic rabbits. In the right panel, the VI is calculated on the basis of the individual histological features. Note that the error bars are standard deviation.

increased macrophage accumulation, less SMC accumulation, and consequently, an increased VI. Our results show that temperature heterogeneity was associated with regions within the plaque of increased MMP-9 activity.

Recently, temperature differences have been detected in explanted human carotid plaques and human coronary arteries *in vivo*.[6,7,9,10] Others and we detected temperature changes in experimentally induced plaques *in vivo* and showed that the presence of macrophages was associated with regional temperature changes.[6,7,9,10] Moreover, discontinuation of the lipid-rich diet resulted in a significant reduction of macrophages, which was associated with the disappearance of the temperature heterogeneity. The present study employed a different method for plaque induction—denudation of the infrarenal aorta—than previous studies, but it identified a similar relationship between macrophage accumulation and temperature increments. The present study shows that the temperature heterogeneity is accompanied by a higher VI and a higher MMP-9 activity.

Surprisingly, no relationship was found between temperature heterogeneity and IVUS-derived plaque thickness. This observation has been shown before[9] and may be the result of an accumulation of macrophages at locations upstream of the plaque,[18] i.e. at locations away from maximal plaque thickness.

The VI has been recently proposed as a histological marker for an inflammatory component of atherosclerosis.[16] It incorporates the cumulative effects of known histological risk factors[19,20] and may, therefore, be a surrogate to test other techniques. The reason for the increase of this index in our animal model was predominantly driven by an increase in macrophage accumulation and a decrease in SMC accumulation. Therefore, these findings suggest that the increased metabolism of active macrophages might explain the increase in temperature in these regions.

Matrix MMPs are a family of enzymes and MMP-2 and MMP-9 belong to the group of gelatinases, which are mainly involved in the migration of cells through the vessel wall, and produced by macrophages, leucocytes, and endothelial cells.[4,11,21] They both are expressed in plaques and MMP-9 has been specifically associated with plaque vulnerability.[4,22,23] Inactive MMP-2 reflects the accumulation of MMP-2 secreting cells, most probably macrophages. Active MMP-2 reflects the activation of these cells in response to the plaque development. The differences between those values may signify the state of activation of these cells. In contrast, MMP-9 values were increased in the regions of increased temperature heterogeneity, which may be explained by the accumulation of macrophages in hot regions. The coincidence of high MMP-9 activity with elevated temperature and vulnerability in this model is in accordance with differential upregulation of MMP-9 in unstable human coronary lesions and plasma of patients with acute coronary syndromes.[11] MMP-2 activity is high in the entire denuded region and only weakly correlated with temperature. Therefore, this increase merely signifies atherogenesis.

Limitations

We have used an animal model with a short duration of a high cholesterol diet to induce regions of atherosclerotic plaque formation. The model mimics certain features of the human form of vulnerable plaque, which are inflammation characterized by macrophage accumulation and lack of SMCs, and a high histologically determined VI. This particular model does not include large lipid cores or spontaneous rupture sites and can therefore only be used to study the inflammatory component rather than the lipid components of (vulnerable) plaque.

Stretch and denudation were necessary for plaque induction, thereby introducing a component of injury to the model. However, we feel that injury is of minor importance for the outcomes of the present study, as (i) we compared hot and cold regions of the same denuded region and (ii) in the normocholesterolaemic rabbit, balloon angioplasty of the iliac artery upregulates MMP-2 and induces its activity, whereas MMP-9 remains below detection limits after 2 months of healing.[24–27] Similar results were found in hypercholesterolaemic rabbits.[28]

In order to increase temperature heterogeneity, we inhibited blood flow through the aorta during the temperature

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measurements. Therefore, extrapolation of these findings to clinical conditions needs to be performed with caution.

In conclusion, *in vivo* temperature measurements under well-controlled experimental conditions enable to detect increased vulnerability, as determined by increased macrophage infiltration and MMP-9 activity. As these phenomena have been associated with plaque vulnerability, these findings may implicate an inflammatory mechanism underlying the high-risk plaque.

Acknowledgements

R.K. is a recipient of the 'Established Investigator Grant' of the Dutch Heart Foundation (grant no. 2002T045).

Conflict of interest: none declared.

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