Decreased Smooth Muscle Cell/Extracellular Matrix Ratio of Media of Femoral Artery in Patients With Atherosclerosis and Hyperhomocysteinemia

E.G.J. Vermeulen, H.W.M. Niessen, M. Bogels, C.D.A. Stehouwer, J.A. Rauwerda, V.W.M. van Hinsbergh

Abstract—The aim of this study was to determine whether the morphology of the muscular femoral artery in patients with atherosclerosis and hyperhomocysteinemia differs from that of atherosclerotic vessels from patients with normal homocysteine levels. Whole-vessel biopsies of the superficial femoral artery were taken from patients with symptomatic atherosclerotic disease with and without hyperhomocysteinemia and from patients without atherosclerosis from traumatic amputations. The morphology of these specimens was studied qualitatively by light and electron microscopy and quantitatively by light microscopy in combination with a video overlay system. Atherosclerotic lesions in patients with hyperhomocysteinemia were morphologically similar to those in patients with normal homocysteine levels, except for a significantly decreased smooth muscle cell/extracellular matrix ratio of the media in hyperhomocysteinemic patients ($P < 0.02$ versus normohomocysteinemic atherosclerotic group and $P < 0.001$ versus group without a history of cardiovascular disease). Hyperhomocysteinemia is associated with a significant decrease of the smooth muscle cell/extracellular matrix ratio of the media of muscular femoral arteries without significant changes in medial thickness. Further investigations should concentrate on the cause of this newly discovered phenomenon and its impact on vascular compliance. (Arterioscler Thromb Vasc Biol. 2001;21:573-577.)

Key Words: hyperhomocysteinemia ■ smooth muscle cells ■ arterial wall histology ■ extracellular matrix

The hypothesis that hyperhomocysteinemia is a risk factor for atherosclerosis was first proposed by McCully, who observed premature atherosclerosis in children with rare metabolic disorders causing markedly elevated levels of plasma homocysteine. Subsequent investigations confirmed this hypothesis, and it has become clear that hyperhomocysteinemia is an independent risk factor for atherosclerotic disease.

Pathophysiological observations in animals and humans have led to the formulation of the response-to-injury hypothesis of atherosclerosis. Arterial lesions observed in patients with severe hyperhomocysteinemia are characterized by proliferative fibrous intimal plaque, disorganization, and fibrosis of the media and by other extracellular matrix (ECM) alterations. These observations and findings in animal models of hyperhomocysteinemia show that vascular changes also occur in the medial layer of atherosclerotic arteries and concern smooth muscle cells (SMCs) and ECM.

Because there are no published human studies concerning the morphology of muscular arteries in the case of mild to moderate hyperhomocysteinemia, we investigated the vascular changes associated with hyperhomocysteinemia in the intimal and medial layers of the superficial femoral artery, a muscular artery prone to atherosclerosis.

Methods

Vascular Biopsies

From 1992 until 1998, whole-vessel biopsies (length 1 to 3 cm) were taken from occluded superficial femoral arteries from patients undergoing bypass graft surgery for symptomatic occlusive peripheral vascular disease. Symptoms consisted of severe ischemic rest pain or gangrene (Fontaine stages III to IV or Rutherford grades II to III).

According to preoperative tests, 6 subjects had hyperhomocysteinemia and 6 had normal homocysteine levels, as defined below. In addition, superficial femoral arterial biopsies were taken from 3 patients suffering traumatic leg amputation who had no history of cardiovascular disease; their homocysteine levels were unknown. The present study was approved by the ethical review committee of the University Hospital “Vrije Universiteit,” Amsterdam, and informed consent was obtained from all subjects.

Processing of Tissue Specimens

The specimens were routinely fixed in 4% formalin and subsequently embedded in paraffin. Paraffin-embedded vascular tissue sections (4 µm) were mounted on microscope slides, deparaffinized for 10 minutes in xylene at room temperature, and rehydrated through...
descending concentrations of ethanol. Sections were then stained with hematoxylin-eosin, elastica van Gieson, and Alcian blue. The morphology was qualitatively evaluated by light microscopy with the use of the classification of the American Heart Association.10

**Immunohistochemistry**

Subsequent to deparaffinization and rehydration, sections were treated with 0.3% H2O2 in methanol for 30 minutes to block endogenous peroxidase activity. Sections then were preincubated with normal rabbit serum (1:50, Dako A/S) for 10 minutes at room temperature and incubated for 60 minutes with anti-α-SMC actin antibody (SMA, 1:200 Dako A/S). After a wash in PBS, sections were incubated for 30 minutes with rabbit anti-mouse biotin-labeled antibody (1:500) at room temperature and subsequently washed in PBS. After incubation with biotin-labeled streptavidin–horseradish peroxidase (1:200, Dako A/S) for 60 minutes at room temperature, horseradish peroxidase was visualized with 3,3′-diaminobenzidine tetrahydrochloride/H2O2 (Sigma Chemical Co) for 3 to 5 minutes.

**Immunoscoring and Immunounoquantification**

SMC/ECM ratios of the intimal and the medial layers were initially determined visually in a microscopic study of the morphology of the vascular biopsies. Further quantification of these ratios in both layers was performed by using a video overlay system (QPRODIT 5.2, Leica).11 From the immunohistochemical staining, the positively stained SMA was taken as a measure of SMCs, and the nonstaining part was used as a measure of ECM. The lamina elastica interna was taken as the border between the intimal and the medial layer. Scoring was performed on 3 random slices of each specimen by 1 pathologist blinded with regard to the homocysteine levels and other clinical data.

The video overlay system was composed of a microscope equipped with a motorized scanning stage and was connected to a computer, which displayed these images in a software environment to interact with the images. After marking an area of interest, ie, the intima or media, the software automatically divided the area into 50 fields of vision. The amount of SMA in either the media or the intima was determined by means of the 2-class immunoscoring module of the QPRODIT system. The amount of SMA was determined by using a point-sampling method with a point grid that was displayed on the screen and superimposed on the projected image of the tissue. The point grid consisted of 12 points, which was systematically displayed over each field of vision. If 1 of those 12 points was within an SMC (colored), it was scored as a positive hit, and if a point was within the ECM (colorless), it was scored as a negative hit. The SMC/ECM ratio was then determined by dividing the amount of SMA by the amount of ECM. Sections were scored by using a ×400 magnification. The mean thickness of the medial layer throughout the specimen was determined by the QPRODIT system at 5 sites randomly. Sites at which the atheroma eroded into the medial layer were excluded.

**Extracellular Matrix**

The ECM of the medial layer was investigated qualitatively for its different components by electron microscopy. Therefore, part of the arterial biopsies were fixed in 2% (vol/vol) glutaraldehyde for 30 minutes and 1.5% (vol/vol) osmium tetroxide for 10 minutes, dehydrated with acetone, and embedded in Epon 812. Ultrathin sections were then collected on 300-mesh nickel grids coated with Formvar (Monsanto). The sections were contrasted with uranyl acetate and lead citrate. Subsequent transmural sections were examined in a JEOL 1200EX electron microscope (JEOL Ltd).

**Methionine Loading Test and Other Clinical Data**

A fasting venous blood sample was taken at 9:00 AM, and a second blood sample was obtained 6 hours after an oral methionine load (0.1 g/kg body wt). Total (free plus protein bound) homocysteine concentrations were measured by using high-pressure liquid chromatography with fluorescence detection.12 Reference values (ie, values ±2 SD above the mean of apparently healthy control subjects) for respective fasting and postmethionine homocysteine levels in our laboratory are <18 and <54 μmol/L in men, <15 and <51 μmol/L in premenopausal women, and <19 and <69 μmol/L in postmenopausal women.13 Patients were considered hyperhomocysteinemic if fasting and/or postmethionine homocysteine levels were above these reference values. None of the patients received vitamin treatment before surgery.

Hypertension was defined as systolic blood pressure ≥160 mm Hg, diastolic blood pressure ≥95 mm Hg, and/or the use of antihypertensive drugs. Diabetes mellitus was defined according to World Health Organization (1985) criteria. Smoking was defined as currently smoking ≥1 cigarette, pipe, or cigar per day. Serum total cholesterol was measured by routine methods.

**Statistical Analysis**

Data are given as median (range), as mean±SD, or as numbers. Skewed data were logarithmically transformed. Continuous variables were tested by Student t test (for means), and other variables were tested by χ² tests with the use of SPSS (version 9.0). In all tests, a 5% significance level was used.

**Results**

The demographics of the patients are shown in the Table. Fasting (P=0.07) and postload (P=0.01) homocysteine levels showed a difference between the normohomocysteinemic and hyperhomocysteinemic atherosclerotic group. There were no other significant differences among the groups.

**Morphology of the Biopsies**

All subjects in the 2 groups with clinical vascular disease showed advanced lesions of atherosclerosis (types V and VI by American Heart Association classification). The subjects in the control group were without signs of atherosclerosis. There were no obvious differences between the atherosclerotic groups with regard to occlusion of the lumen, thrombosis, and inflammatory infiltrate (histiocytes and lymphocytes), neovessels in the media, calcification in the intima or media, or deposition of mucopolysaccharides (Figures 1 and 2; other data not shown).

In the biopsies from patients with atherosclerosis with or without hyperhomocysteinemia, elastic fibers in the media did not differ with regard to amount, pattern, or splicing. The internal and external elastic layers were fragmented, partly absent, and partly spliced in all atherosclerotic lesions, independent of homocysteine status. In the subjects without atherosclerosis, these elastic layers were only minimally affected (data not shown).

Electron microscopy showed that the ECM in the atherosclerotic lesions consisted of collagen fibers and glycosaminoglycans (Figure I; please see http://atvb.ahajournals.org). There were no apparent differences in collagen and glycosaminoglycans of the ECM in the media between both atherosclerotic groups. In the vascular tissue of subjects without a history of cardiovascular disease, collagen degradation was minimal (data not shown).

**Quantification of Intimal and Medial SMCs**

Because in both atherosclerotic groups the majority of the arterial specimens were occluded, the intimal thicknesses could not be determined reliably. The SMC/ECM ratios in the SMC-rich areas of the intimal layer were similar between both atherosclerotic groups (Table). In the medial layer, the ratio of SMC to ECM was clearly diminished in the atherosclerotic groups versus the group without a history of cardiovascular disease (Figure 3). Quantification (see Methods) of the SMC/ECM ratio in the media showed a significant
decrease in the hyperhomocysteinemic and the normohomo-
cysteinemic atherosclerotic group versus the group without a
history of cardiovascular disease (P<0.001 and P<0.03,
respectively; Table). The SMC/ECM ratio was significantly
lower in the hyperhomocysteinemic than in the normohomo-
cysteinemic atherosclerotic group (P<0.02, Table). No dif-
ferences were seen among the groups with regard to the mean
thickness of the medial layer (Table).

Discussion
In the present study, atherosclerotic femoral arteries, com-
pared with nonatherosclerotic femoral arteries, showed a
decreased SMC/ECM ratio of the medial layer. The novel
finding in the present study is that this decreased SMC/ECM
ratio was more apparent in the group with atherosclerosis and
hyperhomocysteinemia than in the group with atherosclerosis
and normal homocysteine levels. Although based on a limited
number of specimens and potentially not taken from identical
anatomic sites, these data suggest that elevated concentrations
of homocysteine affect not only the intimal layer but also the
medial layer of muscular arteries. These findings in human
specimens are consistent with findings in animal
experiments.8

Because the European Concerted Action Project (CO-
MAC) study showed that the associations between cardiovas-
cular disease and fasting or postmethionine concentrations of
homocysteine are of similar strength and are mutually inde-
pendent,5 we included patients on the basis of whether or not

Patient Characteristics and Morphometric Quantities According to Group and Homocysteine Levels

<table>
<thead>
<tr>
<th>Group</th>
<th>Subjects Without History of Cardiovascular Disease (N=3)</th>
<th>Atherosclerotic Groups</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Median age, y] 24 (19–71)</td>
<td>[54 (36–70)]</td>
<td>[44 (19–45)*]</td>
</tr>
<tr>
<td></td>
<td>[Male/female, n/n] 3/0</td>
<td>[4/2]</td>
<td>[3/3]</td>
</tr>
<tr>
<td>Fasting tHcy, μmol/L</td>
<td>[7.2±1.5]</td>
<td>[18.1±8.6*]</td>
<td></td>
</tr>
<tr>
<td>Postload tHcy, μmol/L</td>
<td>[28.5±4.7]</td>
<td>[54.4±12.9†]</td>
<td></td>
</tr>
<tr>
<td>Smoking (yes), n</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Diabetes mellitus (yes), n</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension (yes), n</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>4.9±2.2</td>
<td>6.1±1.6</td>
<td>5.5±1.7</td>
</tr>
<tr>
<td>SMC/ECM ratio in intima</td>
<td>31.9±24.8</td>
<td>27.2±3.0</td>
<td></td>
</tr>
<tr>
<td>SMC/ECM ratio in media</td>
<td>0.72±0.08</td>
<td>0.53±0.11†</td>
<td>0.34±0.11§</td>
</tr>
<tr>
<td>Mean medial thickness, μm</td>
<td>590±274</td>
<td>687±466</td>
<td>587±185</td>
</tr>
</tbody>
</table>

Values are median (range), mean±SD, or number. tHcy indicates total serum homocysteine. *P<0.05, †P<0.01 vs normohomocysteinemic atherosclerotic group; ‡P=0.03 vs subjects without a history of cardiovascular disease; §P=0.02 vs atherosclerotic normohomocysteinemic group; and ||P=0.001 vs subjects without a history of cardiovascular disease. All other comparisons were nonsignificant (P>0.10).

Figure 1. Intimal morphology (i indicates intimal layer; m, medial layer). Hematoxylin-eosin staining is shown in the intima (original magnification ×100) of an atherosclerotic femoral artery of a hyperhomocysteinemic patient (hhc) and an atherosclerotic femoral artery of a normohomo-
cysteinemic patient (non-hhc). There are no apparent differences.
they had high total homocysteine concentrations in the fasting state or after methionine loading. Although determination of total homocysteine concentrations is a routine part of the premature atherosclerosis protocol of the vascular surgical unit, it is not performed in patients suffering traumatic amputation; therefore, no homocysteine concentrations are available for the control group. Apart from the total homocysteine concentrations, there are no statistical differences in the baseline characteristics among the groups (Table), but it is likely that some of the differences between the atherosclerotic groups and the traumatic amputation group are related to differences in age and exposure to risk factors such as smoking and dyslipidemia. However, the atherosclerotic groups were similar in these respects; therefore, we conclude that it is likely that the histological differences between these groups were related to their homocysteine levels.

The constant mean thickness of the medial layer throughout the specimens suggests a decreased number of SMCs with a comparable increase in volume of the ECM. Although homocysteine is thought to injure endothelium, to stimulate the proliferation of SMCs, and to induce collagen expression in SMCs in vitro, it cannot be concluded from our data whether stimulated synthesis of ECM caused the decreased number of SMCs or whether a primary decrease in the number of SMCs was substituted by additional ECM. The
examination of the ECM by electron microscopy revealed loss of collagen and accumulation of glycosaminoglycans in both atherosclerotic groups. However, this only demonstrates the existence of collagen degradation and does not allow conclusions about the balance between collagen synthesis and degradation.

The pathophysiological impact of a changed SMC/ECM ratio in the medial layer of muscular arteries is not clear but might result in an altered, possibly decreased vascular elastic compliance and thus increase systolic blood pressure and cardiac afterload.9,16,19–21 However, in vivo data, are not consistent in this respect.22 The mechanisms by which hyperhomocysteinemia is related to a decreased SMC/ECM ratio in the media also require further study.

In conclusion, the present study showed that in atherosclerotic hyperhomocysteinemic patients, the SMC/ECM ratio in the medial layer of the femoral artery was significantly decreased compared with that in patients without atherosclerosis and in patients with atherosclerosis but without hyperhomocysteinemia. Forthcoming investigations should concentrate on the cause of this new phenomenon and its impact on vascular compliance.

Acknowledgments

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References

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