Accumulation of Zinc in Human Atherosclerotic Lesions Correlates With Calcium Levels but Does Not Protect Against Protein Oxidation

Nadina Stadler, Naomi Stanley, Sylvia Heeneman, Vladimir Vacata, Mat J.A.P. Daemen, Paul G. Bannon, Johannes Waltenberger, Michael J. Davies

Objective—Oxidized lipids and proteins, and decreased antioxidant levels, have been detected in human atherosclerotic lesions, with oxidation catalyzed by iron and copper postulated to contribute to lesion development. Zinc has been postulated to displace iron from critical sites and thereby protect against damage. In this study, metal ion and protein oxidation levels were quantified in human carotid and abdominal artery specimens containing early to advanced lesions, to determine whether zinc concentrations correlate inversely with iron levels and protein oxidation.

Methods and Results—Metal ions were quantified by EPR and inductively coupled plasma mass spectroscopy. Native and oxidized protein side-chains were quantified by high-performance liquid chromatography. Elevated levels of zinc (≈6-fold) were detected in advanced lesions compared to healthy tissue or early lesions. Zinc did not correlate negatively with iron or copper levels suggesting that zinc does not displace these metal ions. Highly significant positive correlations (P<0.005) were detected between zinc and calcium levels.

Conclusions—Zinc did not correlate with low iron levels and reduced protein oxidation. These data indicate that zinc does not prevent protein oxidation in advanced lesions. The reported protective effect of zinc accumulation is proposed to be associated with lesion calcification. (Arterioscler Thromb Vasc Biol 2008;28:000-000.)

Key Words: atherosclerosis ■ iron ■ zinc ■ protein oxidation ■ calcium

Atherosclerosis is a multifactorial disease to which many factors contribute; defining the role of each of these has proved to be problematic. Oxidation, and in particular that of low-density lipoproteins (LDL), has been linked to disease development, although the significance of this process remains to be fully established (reviewed). Previous studies have variously implicated lipoxygenase, peroxynitrite, myeloperoxidase, oxygen radicals, and metal-ions in lesion oxidation (reviewed). In the case of metal ions, a correlation between iron accumulation and the extent of protein (but not lipid) oxidation has been reported for human lesions. Cholesterol ester and cholesterol accumulation also correlate positively with iron, suggesting that these processes are interlinked. These data are consistent with some, but not all, epidemiological studies on the potential links between iron and cardiovascular disease.

Zinc ions have been reported to modulate oxidant damage, via the displacement of iron and copper from oxidation-sensitive sites on erythrocyte membranes, LDL, or liposomes. Epidemiological data indicate that elevated zinc levels may be protective against disease. Serum or plasma measurements of zinc in people with established atherosclerosis indicate that low zinc levels are associated with increased disease, and a postmortem study has reported lower tissue levels of zinc in the abdominal aorta of patients who died of heart disease compared to other causes.

In contrast, zinc supplementation of the human diet does not affect the susceptibility of LDL to oxidation ex vivo or the concentrations of LDL-cholesterol, total cholesterol, or triglycerides, but has an adverse effect on high-density lipoprotein levels, suggesting that high serum zinc levels may promote disease. Elevated zinc may stimulate the formation of oxidants and inhibit protective enzymes in some cells.

Atherosclerotic lesions of cholesterol-fed rabbits contain elevated levels of iron and reduced levels of zinc. Zinc supplementation, via dietary feeding, reduced lesion area, despite insignificant changes in lesion zinc concentrations; these changes were ascribed to a displacement of iron by zinc. Zinc supplementation was also shown to reduce accumulation of cholesterol in the aorta, decrease the average...
aortic lesion cross-sectional area, and reduce a number of markers of cholesterol and lipid oxidation. These data are consistent with zinc having an antiatherogenic effect, with this postulated to occur via a reduction in iron-catalyzed radical reactions. High zinc concentrations have also been reported to reduce intimal hyperplasia in a rat carotid artery balloon-injury model.

Zinc may modulate biological effects via a range of alternative mechanisms than prevention of oxidation, such as via the stabilization of zinc-finger genes involved in lipid metabolism, via peroxisome proliferator activated receptor signaling, by modulating transcription factors such as NF-κB and AP-1, and by interference with caspase expression and apoptosis.

In the light of this conflicting data on the role of zinc in atherosclerosis and uncertainty about the mechanism of the reported effects, we investigated the levels of zinc, iron, calcium, and copper in carotid and abdominal atherosclerotic lesions (both postmortem and from endarterectomy operations) and examined whether metal ion levels correlate with the extent of protein oxidation.

Methods

Postmortem Tissue Specimens

Fifty-three human specimens (26 carotid artery, Car; 27 abdominal aorta, AbAo) were obtained from 16 donors aged 53 to 91, autopsied at the University Hospital Maastricht, via the Maastricht Pathology Tissue Collection. The collection, storage, and use of tissue and patient data were performed in accordance with the "Code for Proper Use of Secondary Human Tissue in the Netherlands." In most cases, 2×1 cm consecutive ring segments of the common right Car (1.5 cm proximal of the carotid bifurcation) and 2×2 cm segments of AbAo were removed and rinsed. Four-mm slices from the end of segment were cut on ice, fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, stained, and classified according to Virmani et al. Remaining tissue was stored at 80°C. Sections were classified as: nonulcerated, early nonatherosclerotic intimal lesions (intimal thickening and intimal xanthoma; INI); intermediate progressive atherosclerotic lesions (pathological intimal thickening; PTT); and advanced lesions (ADV) characterized by thick or thin fibrous cap atheromas containing either a well-formed necrotic core, calcification, fibrous tissue, or lesions containing a thrombus, which included ruptured plaques, or those with intraplaque hemorrhage.

Carotid Endarterectomy Specimens

Forty-four human artery samples were obtained after informed consent and ethics committee approval (Royal Prince Alfred Hospital, Sydney, Australia) from symptomatic patients undergoing carotid endarterectomy operations. Twelve mammary and 4 radial artery specimens (from bypass and transplantation operations) were used as control tissue, because of the nonavailability of healthy Car samples. Samples were stored and processed as previously.

Electron Paramagnetic Resonance Spectroscopy

Iron levels were analyzed, nondestructively, by electron paramagnetic resonance (EPR) at 77 K. Sections were classified as: nonulcerated, early nonatherosclerotic intimal lesions (intimal thickening and intimal xanthoma; INI); intermediate progressive atherosclerotic lesions (pathological intimal thickening; PTT); and advanced lesions (ADV) characterized by thick or thin fibrous cap atheromas containing either a well-formed necrotic core, calcification, fibrous tissue, or lesions containing a thrombus, which included ruptured plaques, or those with intraplaque hemorrhage.

Electronic Coupled Plasma Mass Spectroscopy

Samples were analyzed for total iron, calcium, zinc, and copper as previously.

Quantification of Protein-Bound Amino Acids and Oxidation Products

Tissue samples were hydrolyzed to free amino acids and analyzed by HPLC with UV and fluorescence detection as described previously. Protein levels were quantified on homogenates.

Statistical Analyses

Data and bivariate correlation analysis was performed using Xtract (© Dr Vladimir Vacata) and Prism (Version 4.0a for Macintosh, GraphPad Software). Correlations are expressed as Pearson correlation coefficients. Mann–Whitney 1-way ANOVA with Tukey’s post hoc multiple comparison tests were used for metal ion and oxidized amino acid levels. P<0.05 was considered significant.

Results

Donor Characteristics and Artery Classification

Clinical characteristics and sample numbers are presented in supplemental Table I (available online at http://atvb.ahajournals.org). For the postmortem samples, the donor’s clinical record was reviewed for risk factors and cardiovascular events. Two independent investigators, blinded to clinical status, performed the histological analysis. One hundred one samples from 32 donors were initially examined; samples with inhomogeneous histomorphological classification. Type 2 diabetes, unknown glycaemic status, and elevated fasting glucose levels were excluded. To allow stratification of data, cardiovascular events or symptoms indicative of disease including angina pectoris, dyspnea, myocardial infarction, atherosclerosis, transient ischemic attack, and cerebrovascular ischemic stroke were considered.

Quantification of Iron Levels in Artery Samples by EPR Spectroscopy

All lesions gave EPR absorptions at g ~4, characteristic of high spin, rhombic, mononuclear Fe(III) complexes as previously. This signal, which was not detected in control tubes, is distinct from that of Fe(III) heme proteins which have g ~2. Additional EPR absorptions were detected around g ~2, characteristic of organic radicals or iron sulfur clusters; these signals were not investigated further. The g ~4 signal was quantified by double integration of the signal and comparison with standard curves generated using Fe(III)-desferrioxamine. For the postmortem abdominal samples, iron concentrations increased with lesion severity, with significantly higher levels of iron detected in the advanced AbAo compared to Car lesions (Figure 1A). No data for early AbAo lesions was obtained because of low sample numbers. For the corresponding Car samples, there was no significant difference in the level of EPR-detectable iron between the early and advanced lesions (Figure 1A). The Car endarterectomy (CEA) samples, which were all categorized as advanced lesions, contained significantly more EPR-detectable iron than corresponding Car postmortem samples. The concentrations detected in these lesions are similar to those reported previously, with these values being significantly elevated compared to those from healthy artery tissue (Figure 1A).

Quantification of Metal Ion Levels in Artery Samples by Inductively Coupled Plasma Mass Spectroscopy

Total lesion iron, copper, calcium and zinc concentrations were determined by inductively coupled plasma mass spectroscopy (ICPMS; Figure 1). The iron levels were significantly higher than those detected by EPR, as expected, but the trends were similar. There was an increase in iron levels with lesion severity in the abdominal samples, and high levels...
of iron were also detected in the advanced Car specimens. The Car endarterectomy samples contained significantly more iron than the corresponding postmortem Car initial (P=0.0017) or advanced (P=0.024) lesions.

Zinc and calcium concentrations followed a similar pattern to that detected for iron, with higher levels detected in the more advanced lesions, irrespective of AbAo or Car origin. The levels in the Car endarterectomy samples were higher than the postmortem samples (Figure 1B). Copper concentrations did not vary significantly between the various Car specimens (Figure 1C), and the levels of this metal ion were considerably lower than for iron. Calcium levels were significantly elevated (P=0.007) in the Car endarterectomy samples compared to the postmortem initial Car samples. With the exception of copper, the levels of iron, calcium, and zinc measured in the healthy artery samples and initial, preatherosclerotic, Car lesions were similar, and lower than in their advanced counterparts. The detected metal ion concentrations did not correlate with age of the tissue donor (data not shown).

**Quantification of Protein Oxidation Products in Artery Samples**

We have previously reported elevated levels of protein side-chain oxidation products in advanced Car endarterectomy samples compared to healthy tissue.3,30 For the postmortem Car samples, no significant differences were detected between early and advanced lesions for the 4 protein oxida-
tion markers examined (Table 1). The levels of protein-bound o-Tyr detected in the Car endarterectomy samples were significantly greater than in the initial and advanced postmortem Car samples ($P=0.002$ and $0.0142$, respectively), $m$-Tyr levels were significantly elevated in the endarterectomy versus the initial postmortem Car samples ($P=0.0089$), and DOPA levels were significantly elevated in the Car endarterectomy samples when compared to both initial ($P=0.0017$) and advanced postmortem Car lesions ($P=0.0048$). A gender-dependent difference was detected in the di-Tyr levels measured in the initial postmortem Car lesions ($P=0.027$).

Correlation Between Zinc Levels and Other Metal Ions in Lesions

For the postmortem AbAo samples the zinc levels did not correlate significantly with either EPR-detectable iron ($r=0.105$, $P=0.25$) or total iron levels ($r=0.161$, $P=0.25$) (data not shown). In contrast, highly-significant positive correlations were detected between zinc and copper concentrations ($r=0.891$, $P=0.005$) and calcium ($r=0.899$, $P=0.0005$; Figure 2 A1 and B1, respectively). A similar pattern was observed with the postmortem Car lesions, with a significant correlation with copper ($r=0.438$, $P=0.02$, Figure 2 A2), a highly-significant positive correlation detected between zinc and calcium concentrations ($r=0.741$, $P=0.005$; Figure 2 B2), but no correlation with either EPR-detectable iron ($r=0.129$, $P=0.20$) or total iron levels ($r=0.136$, $P=0.25$; data not shown). The Car endarterectomy samples showed a similar pattern, with significant correlations between zinc and calcium ($r=0.895$, $P=0.0001$; Figure 2 B3), zinc and copper levels ($r=0.363$, $P=0.015$; Figure 2 A3), no correlation with total iron levels ($r=0.2173$, $P=0.154$; data not shown), but a positive correlation between zinc with EPR-detectable iron ($r=0.468$, $P=0.0013$; data not shown).

Correlation Between Zinc Levels and Protein Oxidation Parameters in Lesions

For the AbAo samples no significant correlation was observed between zinc levels and any of the 4 markers of protein oxidation: DOPA, $m$-Tyr, or di-Tyr (Table 2). This conclusion is also valid for all postmortem Car samples, with no correlation detected for 3 of the protein oxidation markers: DOPA, o-Tyr, or di-Tyr, though a positive correlation was detected between zinc and $m$-Tyr levels ($r=0.675$, $P=0.0005$). With the Car endarterectomy samples no correlation was observed between zinc and any of the protein oxidation markers (Table 2).

Correlation Between Zinc, Iron, and Protein Oxidation Levels in Lesions Stemming From Postmortem Donors With Diagnosed Heart Disease

The relationship between zinc, protein oxidation, and other metal ions was explored further in 2 populations within the postmortem sample groups: those obtained from subjects that died after an infarction or stroke (9 AbAo, 5 Car samples) and a larger group, including the above, obtained from subjects who died from, or who had been diagnosed with, cardiovascular disease (dyspnea, angina pectoris, atherosclerosis; 18 AbAo and 14 Car). Analysis of the data from the first group resulted in positive correlations for the AbAo lesions between zinc and calcium ($r=0.91$, $P=0.0005$) and zinc and copper ($r=0.512$, $P=0.01$), but all other correlations were insignificant including those between zinc and each measure of iron and between zinc and each marker of protein oxidation. The Car samples were not analyzed because of low numbers.

A similar pattern was detected with the second group of samples. No correlations were detected between zinc and either measure of iron in the AbAo lesions (EPR data, Figure 3 A1; ICPMS data not shown), nor for zinc and any of the markers of protein oxidation. In contrast for the Car lesions a significant negative correlation was detected between zinc and EPR-detectable iron (Figure 3 A2; $r=-0.664$, $P=0.002$), but not for total iron levels. In both the AbAo and Car lesions significant correlations were detected between zinc and calcium ($r=0.908$, $P=0.0005$; $r=0.822$, $P=0.0005$ respectively; Figure 3 B1 and B2) and zinc and copper ($r=0.679$, $P=0.0025$; $r=0.511$, $P=0.05$ respectively; Figure 3 C1 and C2).

The similarity between the correlations observed with these samples from subjects with diagnosed cardiovascular disease, and the entire cohort suggests that the clinical background of the subjects (and particularly previous treatment for the diagnosed condition) does not modify the relationship between zinc and the other parameters measured.
Discussion

Previous studies have reported positive and negative data on the protective effect of zinc against atherosclerosis. It has been proposed that zinc displaces iron and copper from oxidation-vulnerable sites thereby limiting damage. Dietary zinc supplementation in cholesterol-fed rabbits decreases the extent of lesion lipid oxidation and attenuates atherosclerotic burden, despite insignificant changes in lesion zinc. Zinc deficiency in rats has been reported to enhance LDL oxidation in vitro and in LDL-receptor deficient mice to result in increases in plasma lipids and induction of proinflammatory events. However zinc supplementation of apoE-deficient mice on a high-fat high-cholesterol diet did not confer protection, although a hypolipidemic effect was detected, the activity of the antioxidant enzyme superoxide dismutase was elevated, and plasma oxidation was inhibited. In contrast, zinc may enhance atherosclerosis via increased oxidant generation and decreased high-density lipoprotein levels. Zinc may also modulate atherosclerosis, independent of oxidation, through effects on gene stabilization, transcription factor levels, and apoptosis.

In the present study we demonstrate that elevated levels of both zinc and iron are present in advanced Car and AbAo lesions, compared to healthy tissue. The iron levels did not increase in advanced Car lesions, whereas zinc levels did increase.
correlate inversely with zinc, as would be expected if zinc displaced iron, with the exception of EPR-detectable iron in the advanced Car postmortem samples from patients who had died of cardiovascular events. This negative correlation may reflect an alteration in the type of iron present in these lesions, as the total iron levels in these lesions did not correlate with zinc levels. Copper levels did not vary significantly, and no inverse correlation was detected between copper and zinc concentrations; in contrast a positive association was observed. This may be associated with an elevated level of multiple proteins that bind copper or zinc, or enhanced expression of (one or more) proteins that contain both metals (eg, the cytosolic and extracellular forms of Cu/Zn superoxide dismutase). None of the 4 independent markers of protein oxidation examined suggests that zinc is unlikely to protect against transition-metal induced protein oxidation.

In contrast, highly significant correlations were detected between zinc and calcium in all lesions. These metal ions bind to similar ligands in vitro. These data are consistent with an increased availability of metal ion binding sites, potentially including polyanionic glycosaminoglycans and proteoglycans, which have a high affinity for metal ions. It is unclear whether calcium accumulation occurs concurrently with zinc, but the strength of the observed correlations supports this conclusion. It is not possible to ascertain whether calcium and zinc accumulation occurs independently of iron and copper, or whether all of these metal ions accumulate concurrently. Little is known about the requirements and functions of zinc in maintaining the integrity of the vasculature and the vascular endothelium. Modifications in zinc homeostasis may result in changes in the cellular labile zinc pool and subsequent modulation of the function and activity of zinc-requiring proteins and signaling pathways, in endothelial cells. As the number of zinc-binding proteins is large, determining which proteins are responsible will be
onerous. A limitation of this study is that only total levels of the metal ions were quantified, rather than bioavailable levels, and that the lesions examined are heterogeneous in nature.

The data obtained provide a potential explanation for the reported protective effect of elevated zinc against atherosclerosis. It is well established that highly-fibrotic calcified lesions are less prone to rupture than lipid-rich, matrix-poor, lesions. The decreased extent of cardiovascular events in people with high zinc levels may therefore merely be an indicator of calcium accumulation and fibrosis and hence decreased propensity to lesion rupture. Whether high zinc levels are merely a consequence of calcification and the presence of fibrous lesions or can promote the formation of stable lesions is unclear. Thus high zinc levels may promote lesion stability by binding to matrix components and stabilizing lesion structures; this hypothesis would appear to be worthy of further study. If correct, it may offer novel mechanisms of enhancing lesion stability.

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Disclosures
None.

References
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### Supplementary Table I.

Characteristics of lesion samples examined: sample numbers and sample stratification according to gender, artery type, lesion type and clinical characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Abdominal aorta samples</th>
<th>Carotid artery samples</th>
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<tbody>
<tr>
<td>Age of donors (yrs)</td>
<td>77.7 ± 10.9</td>
<td>74.9 ± 12.3</td>
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<tr>
<td>Samples numbers and gender</td>
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<td>26 (11 female, 15 male)</td>
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<td>Donors with myocardial infarction as death cause</td>
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<td>5</td>
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<tr>
<td>Donors with diagnosed heart disease</td>
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<td>14</td>
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<tr>
<td>Smokers</td>
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<tr>
<td>Preatherosclerotic, initial lesions</td>
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<tr>
<td>Endarterectomy samples</td>
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