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Promotion of atherogenesis by copper or iron—Which is more likely?

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Abstract

Iron levels increase in atherosclerotic lesions in cholesterol fed-rabbits and play a role in atherosclerosis. We investigated whether copper also rises. Male New Zealand White rabbits were fed high-cholesterol diets for 8 weeks. After sacrifice, lesion sizes were determined, and elemental analyses of the lesion and unaffected artery wall performed using nuclear microscopy. Unlike iron, lesion copper is decreased by about half compared with the unaffected artery wall, and much less copper than iron is present. Our data suggest that iron may be more likely to play a role in the promotion of atherosclerosis than copper.

Keywords: Iron; Copper; Elemental analysis; Nuclear microscopy

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in arteries [1]. The development of atherosclerosis depends on a balance between proinflammatory stimuli and anti-inflammatory and antioxidant defence mechanisms [2–4]. Iron and copper have both been implicated in their ability to induce free radical-mediated damage by catalyzing hydroxyl radical formation and lipid peroxidation [4]. Our previous work has implicated iron in the development of atherosclerosis in cholesterol-fed rabbits. When New Zealand white rabbits are fed a 1% cholesterol diet to induce atherosclerosis, the lesions contain much more iron than the unaffected vessel wall [5–8]. If iron accumulation is prevented by weekly venesection, atherosclerosis slows down [6]. Similarly, prolonged administration of the iron chelator, Desferrioxamine mesylate (Desferal®), decreases atherosclerosis development [8].

One of the early events in the pathogenesis of atherosclerosis is the oxidation of low density lipoproteins (LDL). Oxidation results in a modified form of LDL that is recognized and taken up by macrophage scavenger receptors resulting in the formation of lipid-laden foam cells that contribute to atherosclerotic lesions [2–4,9]. Oxidation of LDL can be brought about by a variety of reactive species. Gruel samples from human atherosclerotic lesions have been shown to contain both iron and copper in forms that can catalyze free radical formation [10]. Copper is a powerful catalyst of LDL oxidation *in vitro*, often more effective than iron [9]. When copper ions are added to LDL, they can react with the lipid hydroperoxides (LOOH) within the LDL to generate peroxyl ($LOO^{\cdot}$) and alkoxyl ($LO^{\cdot}$) radicals that continue LDL oxidation. Some of the copper within caeruloplasmin may also be able to catalyse LDL oxidation [11].

The purpose of the present paper was to assess the relative amounts of iron and copper in atherosclerotic lesions in order to gain an insight into their possible relative contributions to the development of atherosclerosis in cholesterol-fed rabbits.

Materials and methods

Sample preparation. Seven male New Zealand White rabbits, weighing approximately 2.5 kg, were used for the study. The rabbits were fed a high-cholesterol diet (standard Guinea Pig and Rabbit diet + 1% cholesterol)

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for 8 weeks to induce atherosclerosis. Such a diet raises plasma cholesterol levels from about 1 mM to about 40 mM [7,8]. After sacrifice at 8 weeks, the aortic arch was removed and cut into three segments as previously described [7]. Segments were flushed with deionised water to remove residual blood from the inner artery wall, and the blocks of tissue were then flash frozen in liquid nitrogen. The tissues were stored at −80 °C, and transported on dry ice whenever necessary. Special care was taken to prevent thawing and refreezing and subsequent ice crystal damage. Serial sections of 14 μm thickness were cut using a Leica CM3050S cryostat set at −22 °C with the knife at −17 °C. Sections were picked up on gelatin-coated glass sides for staining. Hematoxylin and Eosin staining was carried out to identify the lesion and artery. To quantify the lesion area, a Carl Zeiss Axiophot 2 image analyzer with KS400 (version 3.18) software, was used. Adjacent unstained sections were picked up on freshly made pioloform film mounted on nuclear microscopy target holders for nuclear microscopy analysis.

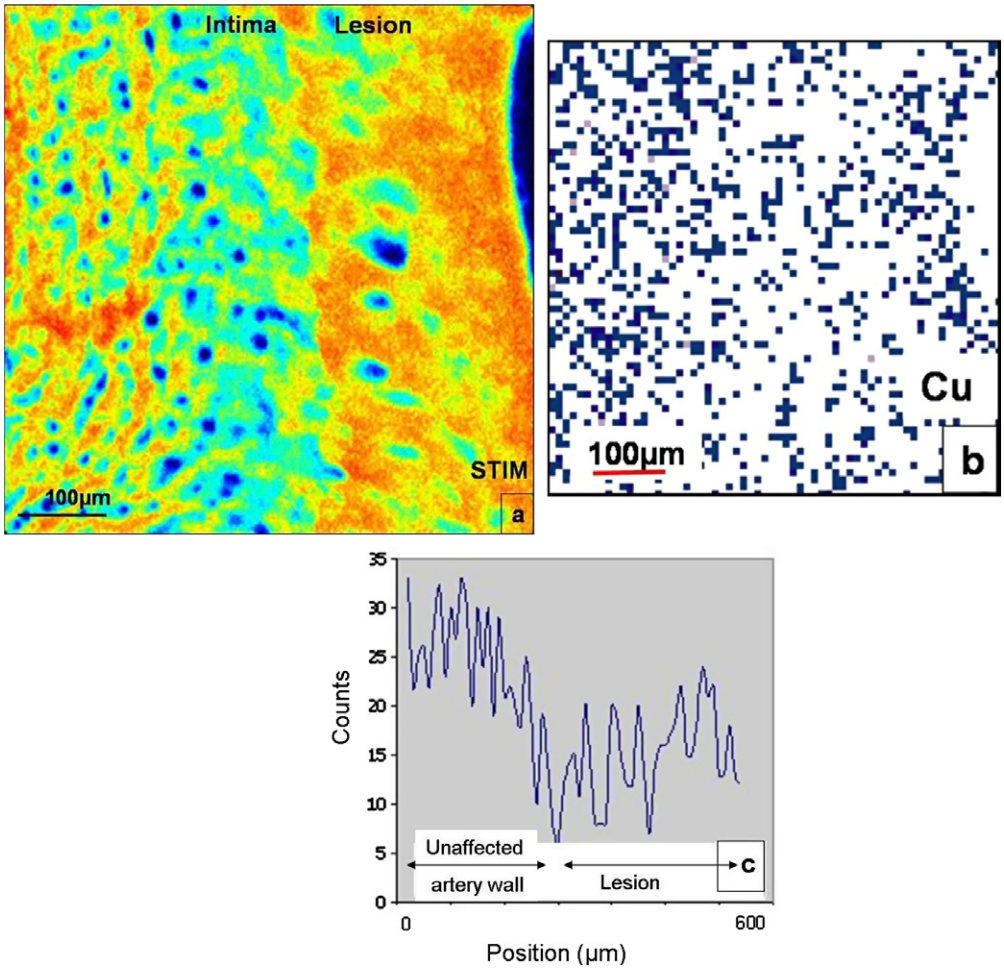

Fig. 1. Atherosclerosis in the cholesterol-fed rabbit. (a) STIM image of part of an artery cross section, showing unaffected artery wall + lesion, (b) representative copper map, of the area, and (c) copper X-ray (PIXE) counts represented as a line scan from right to left across the unaffected wall and lesion. Elemental data extracted from (b and c) show that in this sample the average copper concentration is 2.7 ppm in the lesion and 7.4 ppm in the healthy artery wall.

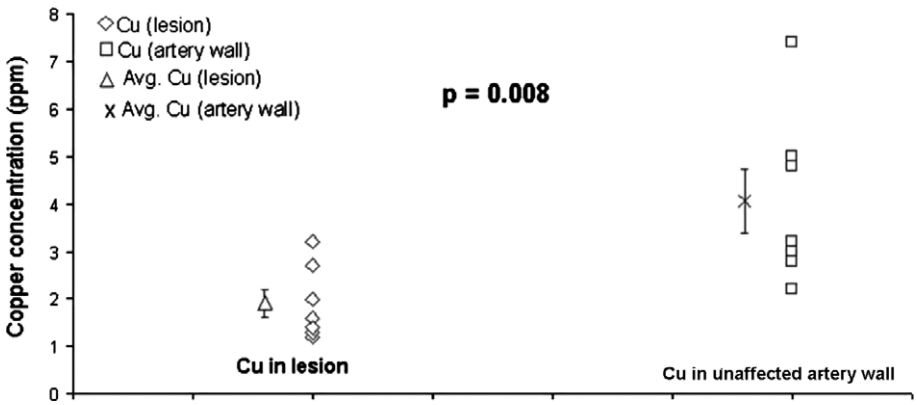

Fig. 2. Atherosclerosis in the cholesterol-fed rabbit. Copper concentrations in lesion and unaffected artery wall.

Nuclear microscopy analysis. The nuclear microscopy experiments were carried out at the Centre for Ion Beam Applications at the National University of Singapore [12]. A 2.1 MeV proton beam focused to a 1 μm spot size was used. Data from the three techniques of Particle-Induced X-Ray Emission (PIXE) for trace elemental analysis, Rutherford Backscattering Spectrometry (RBS) to measure the concentration of matrix constituents of the sample, and Scanning Transmission Ion Microscopy (STIM) to identify regions of interest in the specimen, were simultaneously collected.

Scanning Transmission Ion Microscopy is a technique used to investigate samples which are thin enough for transmission of a 2–3 MeV proton beam. For relatively thin organic samples (30 μm or less), essentially all incident protons that have not suffered nuclear backscattering will pass through the sample. Measurement of the energy or the energy loss of the transmitted proton gives information on the density structure of the sample. PIXE is a well-established technique for trace elemental analysis offering non-destructive multi-elemental capability and low detection limits. It allows simultaneous detection of several elements, with a sensitivity of about 1 ppm in biological material. For analysis of copper, the areas chosen were those with average-sized lesions. As far as possible, care was taken to identify areas with minimum calcification to decrease background and the possibility of errors due to a possible misinterpretation of the PIXE spectra as a Ca pile-up peak.

Statistical analysis. This was carried out using Student's *t*-test, using $p < 0.05$ as a significant difference.

Results

Administration of cholesterol in the diet of rabbits produces a highly reproducible development of atherosclerosis; full details of lesion sizes and elemental distributions can be found in [6–8]. In the present study, measurements were carried out at the lesion/artery wall interface so as to include both the lesion and the unaffected artery wall for comparative analysis (see Fig. 1a).

Copper level: lesion and unaffected artery wall

Area elemental concentration analyses were carried out for both the lesion and artery wall. Because copper is a trace element occurring at concentrations approaching the limit of the PIXE technique, a line scan was also carried out across the lesion and the unaffected artery wall (Fig. 1c). This ensured increased data collection and improved statistics. The line scan across the artery revealed the same trend as the area mapping (Fig. 1b). If copper is involved in atherosclerosis development, we might expect to find higher levels of copper in the lesion compared to the healthy artery wall. However, this was not the case. The average copper levels in the unaffected artery wall were almost double those found in the lesion (4.1 ppm compared with 1.9 ppm—Fig. 2). There was a significant difference ($p = 0.008$) between copper in the lesion and the unaffected artery wall (Fig. 2).

Iron and copper: a comparison

When the levels of copper and iron in the lesion were compared, it was found that the concentration of iron in the lesion was much higher than the quantity of copper.

Table 1
Atherosclerosis in the cholesterol-fed rabbit

	Iron (ppm)		Copper (ppm)		Zinc (ppm)		Fe/Cu ratio		Zn/Cu ratio	
	Lesion	Unaffected artery wall	Lesion	Unaffected artery wall	Lesion	Unaffected artery wall	Lesion	Unaffected artery wall	Lesion	Unaffected artery wall
Animal 1	165.80	39.50	2.70	7.40	55.80	240.40	61.41	5.34	20.67	32.49
Animal 2	31.50	18.90	2.00	2.80	16.80	104.80	15.75	6.75	8.40	37.43
Animal 3	42.30	43.80	3.20	5.00	51.60	118.60	13.22	8.76	16.13	23.72
Animal 4	33.90	16.30	1.20	3.20	21.10	93.80	28.25	5.09	17.58	29.31
Animal 5	86.00	9.00	1.30	3.00	24.20	70.20	66.15	3.00	18.62	23.40
Animal 6	72.70	33.40	1.60	2.20	22.70	93.20	45.44	15.18	14.19	42.36
Animal 7	58.70	17.30	1.40	4.80	27.70	151.80	41.93	3.60	19.79	31.63
Average	70.1 (17.7)	25.5 (5.0)	1.9 (0.29)	4.1 (0.68)	31.4 (5.9)	124.7 (21.5)	38.9 (7.88)	6.82 (1.57)	16.5 (1.58)	31.5 (2.61)
Student's *t*-test	$p = 0.036$		$p = 0.0039$		$p = 0.0019$		$p = 0.0087$		$p = 0.0063$	

Iron, copper, and zinc concentrations and elemental ratios in the lesion and unaffected artery wall for each of the seven animals. The standard errors of the mean are indicated in parenthesis below the averages. A significant difference is observed between the concentrations of iron, copper, and zinc and the ratios of Fe/Cu and Zn/Cu in the lesion and artery which has been calculated using the Student's *t*-test.

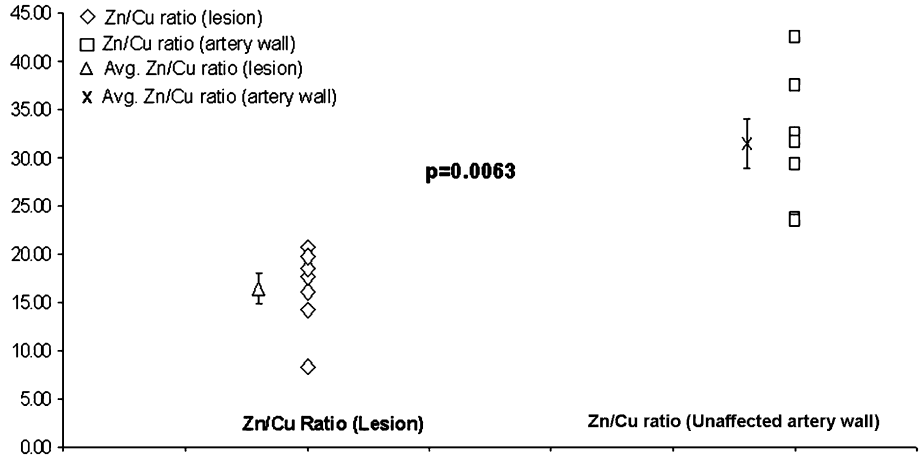

Fig. 3. Atherosclerosis in the cholesterol-fed rabbit. Zn/Cu ratio in lesion and unaffected artery wall.

The mean level of copper in the lesion was 1.9 ppm while the level of iron was 70.1 ppm (Table 1).

Zinc and copper: a comparison

Studies by us and others on New Zealand White rabbits have shown that increased dietary zinc intakes can decrease atherosclerosis [13,14]. However, the exact relationship between the quantities of zinc and copper in blood vessel walls are not known. To understand this better, copper and zinc concentrations were compared. The amounts of copper and zinc appear to be correlated in both the lesion and the healthy artery wall. There seems to be, on average, 16 times more zinc than copper in the lesion and 31 times more zinc than copper in the healthy artery wall (Table 1). The zinc to copper ratio was also significantly different when the lesion was compared with the unaffected artery wall ($p = 0.0063$) (Fig. 3).

Discussion

Previous studies showed that atherosclerotic lesions in cholesterol-fed rabbits contain markedly increased iron concentrations compared with the adjacent unaffected artery wall, implying increased iron-dependent free radical production and possible involvement in the atherosclerotic process. Indeed, the effects of iron chelators and venesection are consistent with this [6–8]. By contrast, our present studies show that copper is decreased in the lesions compared to the healthy artery wall, with a mean value of 1.9 ppm compared with 4.1 ppm. It was also found that the amount of iron in the same lesion was much greater compared to copper (Table 1). This implies that if atherosclerosis involves metals, iron is more likely to play a role than copper because of the greatly increased quantities present.

These results support other studies which suggest that extra dietary copper reduces atherosclerosis in the rabbit [15] and that the probability of iron oxidizing LDL *in vivo* is much more than that of copper [16]. Atherosclerotic lesions may have an acidic extracellular pH, particularly within clusters of macrophages. At acidic pH, LDL oxidation by cells in the presence of iron is increased [16], whereas acidity slows down the initial oxidation in the presence of copper [16,17].

A comparison was also made between the amounts of copper and zinc in the lesions and it was found that, on average, there was 16.5 times more zinc in the lesion than copper (Table 1). Our results show that the relationship between the zinc to copper ratios in both the lesion and the unaffected artery wall (16.5 ± 1.6 and 31.5 ± 2.6) appears closely correlated (Table 1), perhaps implying a chemical link between zinc and copper. A consistent Zn/Cu ratio does not give us any indication of the type of compounds present or the underlying molecular mechanism. It has been reported that CuZnSOD, a known antioxidant, might act as an antioxidant in atherosclerotic lesions [18]. Our studies on Zn/Cu ratios indicate that the measured ratio of 16.5 is greatly different from the approximately 1:1 ratio which would be expected if zinc and copper existed only as CuZnSOD. Thus, while a significant part of the copper might possibly exist in CuZnSOD, most of the zinc must be elsewhere.

Of course, the total levels of metals in a vessel wall are not necessarily equivalent to the levels of redox-active metal ions [4]. When rabbits were administered iron dextran to induce iron overload, atherosclerosis was not accelerated, but the iron contents of the lesion or, healthy vessel wall did not increase (our unpublished data). Thus for example, it might be possible that a greater percentage of the copper present is redox active as compared with iron. However, most studies on the pro-oxidancy of Cu^{2+} towards LDL are done in simple *in vitro* systems. *In vivo*, copper ions can bind actively to many proteins (such as albumin), which can decrease their ability to promote LDL oxidation [4,19]. The large difference in iron and copper levels suggests that iron may be more important, at least in this animal model of atherosclerosis. Indeed copper deficiency has

been linked to increased coronary risk [20], suggesting an antioxidant role of copper *in vivo*. This has been supported by other studies which state that copper deficiency in animals leads to increased lipid peroxidation [21–23], copper deficiency in rats leads to decrease in resistance to oxidation [24], and copper supplementation leads to increased oxidative resistance in a human subgroup [25].

To conclude, we have established that in this animal model, iron in the lesion is pro-atherosclerotic and zinc is anti-atherosclerotic [6–8,14]. Our data in the present paper suggest that copper is more likely to be the latter than the former. The mechanism of copper depletion in the lesions remains to be established. The study was approved by the Institutional Animal Care and Use Committee, NUS.

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References

[1] A.J. Lusis, Atherosclerosis, Nature 407 (2000) 233–241.
[2] American Heart Association, Heart and Stroke Statistical Update, 2001.
[3] R. Stocker, J.F. Keaney Jr., New insights on oxidative stress in the artery wall, J. Thromb. Haemost. 3 (2005) 1825–1834.
[4] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, fourth ed., Oxford University Press, Oxford, 2006.
[5] F. Watt, M. Selley, P.S.P. Thong, S.M. Tang, Nuclear microscopy of atherosclerotic tissue, Nucl. Instrum. Meth. B 104 (1995) 356–360.
[6] D. Ponraj, J. Makjanic, P.S. Thong, B.K. Tan, F. Watt, The onset of atherosclerotic lesion formation in hypercholesterolemic rabbits is delayed by iron depletion, FEBS Lett. 459 (1999) 218–222.
[7] M. Ren, F. Watt, B.T. Huat, B. Halliwell, Correlation of iron and zinc levels with lesion depth in newly formed atherosclerotic lesions, Free Radic. Biol. Med. 34 (2003) 746–752.
[8] M. Ren, R. Rajendran, N. Pan, B.K. Tan, W.Y. Ong, F. Watt, B. Halliwell, The iron chelator desferrioxamine inhibits atherosclerotic lesion development and decreases lesion iron concentrations in the cholesterol-fed rabbit, Free Radic. Biol. Med. 38 (2005) 1206–1211.
[9] H. Esterbauer, J. Gebicki, H. Puhl, G. Jurgens, The role of lipid peroxidation and antioxidants in oxidative modification of LDL, Free Radic. Biol. Med. 13 (1992) 341–390.
[10] C. Smith, M.J. Mitchinson, O.I. Aruoma, B. Halliwell, Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions, Biochem. J. 286 (1992) 901–905.
[11] E. Ehrenwald, P.L. Fox, Role of endogenous ceruloplasmin in low density lipoprotein oxidation by human U937 monocytic cells, J. Clin. Invest. 97 (1996) 884–890.
[12] F. Watt, J.A. van Kan, I. Rajta, A.A. Bettiol, T.F. Choo, M.B.H. Breese, T. Osipowicz, The National University of Singapore high energy ion nano-probe facility: performance tests, Nucl. Instrum. Meth. B 210 (2003) 14–20.
[13] E.M. Alissa, S.M. Bahijri, D.J. Lamb, G.A. Ferns, The effects of coadministration of dietary copper and zinc supplements on atherosclerosis, antioxidant enzymes and indices of lipid peroxidation in the cholesterol-fed rabbit, Int. J. Exp. Pathol. 85 (2004) 265–275.
[14] M. Ren, R. Rajendran, N. Pan, B.K.H. Tan, C.N. Ong, F. Watt, B. Halliwell, Zinc supplementation decreases the development of atherosclerosis in rabbits, Free Radic. Biol. Med. 41 (2006) 222–225.
[15] D.J. Lamb, G.L. Reeves, A. Taylor, G.A. Ferns, Dietary copper supplementation reduces atherosclerosis in the cholesterol-fed rabbit, Atherosclerosis 146 (1999) 33–43.
[16] D.S. Leake, Does an acidic pH explain why low density lipoprotein is oxidised in atherosclerotic lesions? Atherosclerosis 129 (1997) 149–157.
[17] A.J. Rodriguez-Malaver, D.S. Leake, C.A. Rice-Evans, The effects of pH on the oxidation of low-density lipoprotein by copper and metmyoglobin are different, FEBS Lett. 406 (1997) 37–41.
[18] D.J. Lamb, M.L. Tickner, S.M. Hourani, G.A. Ferns, Dietary copper supplements modulate aortic superoxide dismutase, nitric oxide and atherosclerosis, Int. J. Exp. Pathol. 86 (2005) 247–255.
[19] B. Halliwell, J.M. Gutteridge, The antioxidants of human extracellular fluids, Arch. Biochem. Biophys. 280 (1990) 1–8.
[20] G.A. Ferns, D.J. Lamb, A. Taylor, The possible role of copper ions in atherogenesis: the Blue Janus, Atherosclerosis 133 (1997) 139–152.
[21] M. Fields, C.G. Lewis, Benfluorex, a hypotriglyceridemic drug, reduces lipid peroxidation and alleviates adverse metabolic complications of copper deficiency, Nutrition 13 (1997) 895–899.
[22] A. Mazur, E. Gueux, I. Bureau, C. Feillet-Coudray, E. Rock, Y. Rayssiguier, Copper deficiency and lipoprotein oxidation, Atherosclerosis 137 (1998) 443–445.
[23] J.T. Saari, Evidence that dimethyl sulfoxide inhibits defects of copper deficiency by inhibition of glycation, Nutr. Res. 16 (1996) 467–477.
[24] Y. Rayssiguier, E. Gueux, L. Bussiere, A. Mazur, Copper deficiency increases the susceptibility of lipoproteins and tissues to peroxidation in rats, J. Nutr. 123 (1993) 1343–1348.
[25] A.A. Jones, R.A. DiSilvestro, M. Coleman, T.L. Wagner, Copper supplementation of adult men: effects on blood copper enzyme activities and indicators of cardiovascular disease risk, Metabolism 46 (1997) 1380–1383.