Free Rad. Res., Vol. 30, pp. 221–231
Reprints available directly from the publisher
Photocopying permitted by license only

Apoptotic Macrophage-derived Foam Cells of Human Atheromas are Rich in Iron and Ferritin, Suggesting Iron-catalysed Reactions to be Involved in Apoptosis*

XI MING YUAN†

Department of Pathology II and the Clinical Research Center, Faculty of Health Sciences, Linköping University, S-581 85 Linköping, Sweden

Accepted by Prof. B. Halliwell

(Received 14 September 1998; In revised form 20 October 1998)

We investigated the presence of low-molecular-weight iron and ferritin in human atheromas, and their possible relation to the apoptotic process. Arterial wall segments with fatty streaks were collected from coronary arteries and thoracic aortas of 12 clinical autopsy cases with general atherosclerosis. Normal appearing regions from the same cases together with normal coronary arteries from seven young forensic autopsy cases, without any sign of atherosclerosis, were used for comparison. Anti-CD68 (macrophage marker) and anti-ferritin antibodies were applied to serial sections of the arterial wall segments, fixed in formadehyde and embedded in paraffin wax, using an avidin–biotin complex (ABC) technique. Similarly, apoptotic cells were assayed by the TUNEL technique, while low-molecular-weight iron was cytochemically detected by autometallography. Cell counting and computerised image analysis were performed to compare the distribution of macrophages, ferritin- and iron-rich cells, and apoptotic cells in the intima, media, and adventitia of the arteries.

Pronounced ferritin accumulation, occurrence of lysosomal low-molecular-weight iron, and apoptosis mainly concerned CD68-positive cells (macrophages) in the atherosclerotic lesions. No ferritin- or CD68-positivity was found in normal coronary arteries from the young forensic-autopsy cases, while a moderate number of such cells were observed in the intima of normal looking vessel areas from the control cases. In the intima, cytosolic ferritin and low-molecular-weight iron with a lysosomal type distribution were found in many CD68-positive macrophages which frequently were surrounded by erythrocytes. A substantial number of apoptotic cells within the intima, media, and adventitia were registered in all atherosclerotic lesions examined, although mainly in the vulnerable macrophage-enriched areas of the atheroma shoulder.

We suggest that iron may occur within the cytosol, mainly bound in ferritin, but also in low-molecular weight, redox-active form within the acidic vacuolar apparatus of macrophages and macrophage-derived foam cells following erythrophagocytosis or phagocytosis of apoptotic cells. Low-molecular-weight iron within lysosomes, present due to degradation of iron-containing structures, such as ferritin, may partially become exocytosed and contribute to cell-mediated LDL-oxidation. Moreover, such lysosomal iron may also sensitise lysosomes to oxidative stress and induce apoptosis of macrophage/foam-cells that may result in instability and rupture of atherosclerotic plaques.

---

* A portion of these results were presented at the *XIth International Symposium on Atherosclerosis*, Paris, France, October 5–7, 1997 and published in abstract form (*Atherosclerosis* 1997; **134**: 258).

† Tel.: +46 13 224485. Fax: +46 13 221529. E-mail: yuan.ximing@kfc.liu.se.

Keywords: Ferritin, apoptosis, iron, atheroma, macrophages, atherosclerosis

INTRODUCTION

Atherosclerosis causes more deaths than any other single degenerative disease, including cancer. Many clinical and preclinical studies have demonstrated that elevated levels of low density lipoproteins (LDL) may play a critical role at several steps of atherogenesis.[1] Research over the last two decades indicates that oxidative modification of LDL, probably in the arterial intima, is an important and early step in atheroma formation. Exactly how LDL is oxidised remains, however, to be elucidated.[2] One proposed mechanism for this process is transition metal-mediated modification through Fenton-type chemistry.[3,4] It has been found, by us and others, that macrophages, one of the major cellular components of atheromas, are capable of endo- and exocytosing iron compounds, and furthermore, that pronounced iron- and ferritin sequestration occurs in atherosclerotic lesions.[5–7]

Recently, the Bruneck studies on the role of ferritin in atherogenesis have provided additional support to the hypothesis that an elevated amount of body-iron is associated with an increased risk of heart disease.[8] Elevated serum-ferritin was found to be linked to carotid atherosclerosis[9] and considered to be a risk factor in myocardial infarction as well.[10] Under certain conditions ferritin has been suggested to release free, redox-active iron that may initiate LDL oxidation *in vitro*.[11,12] Increased ferritin, ferritin mRNA, and ferritin gene expression have been found in human atherosclerotic lesions. Moreover, ferritin mRNA was markedly increased in the atherosclerotic aortas of rabbits after feeding them a high cholesterol diet for 6 weeks, indicating that iron is sequestered in atheroma cells, and that lipid metabolism interacts with iron handling at the cellular level.[13] However, the implication of such iron-sequestration within macrophages/macrophage-derived foam cells during atherogenesis remains unknown.

Loss, degeneration, and dystrophic calcification of cells in the core regions of atherosclerotic plaques are among the most prominent characteristics in the progression of atherosclerosis, they are also closely linked to plaque instability, rupture, and other complications. Apoptosis of macrophages/macrophage-derived foam cells in atherosclerotic lesions have recently drawn much attention, although the etiological mechanisms of cell death in atherogenesis are still unknown.[14–17] It has, however, been reported that oxidised LDL is cytotoxic and may induce apoptotic death of a variety of cells, including macrophages/foam cells.[18] Recently, we demonstrated that apoptosis of macrophage-derived foam cells *in vitro*, induced by oxidised LDL, begins with partial lysosomal disruption, with ensuing relocation to the cytosol of lysosomal enzymes.[19,20]

This study aimed to investigate the nature of iron-sequestration within macrophages and macrophage-derived foam cells in human atherosclerotic lesions, and its relation to apoptosis and cell death in atherosclerotic lesions.

MATERIALS AND METHODS

1. Arterial Specimens and Sampling

Arterial wall segments with fatty streaks[21] were collected from coronary arteries and thoracic aortas of 12 clinical autopsy cases. Macroscopical normal areas of arteries from the same cases together with normal coronary arteries from seven young forensic autopsy cases, without any evidence of atherosclerosis, were used for comparison with the atherosclerotic lesions. The samples were fixed in 10% formalin in phosphate-buffered saline and embedded in paraffin. Routine haematoxylin and eosin staining were prepared in all cases.

2. Immunohistochemistry and Iron Histochemistry

In order to characterise the nature and location of ferritin/iron accumulation in the vessel specimens, monoclonal anti-CD68 antibodies, a human macrophage marker, and polyclonal rabbit anti-human ferritin antibodies were applied to serial sections using the streptavidin–biotin complex (ABC) technique. The sections were prepared according to the handbook of immunochemical staining methods from DAKO corporation. To block endogenous peroxidase, and improve availability, the sections were treated with 3% H_2O_2 for 5 min (CD68 and ferritin) and trypsin (CD68) for 10 min at 37°C. Trypsin/H_2O_2-treatment was not performed for ferritin immunohistochemistry. Sections were washed, and blocked with normal swine-serum, and incubated with the antibodies (anti-ferritin and anti-CD68). Biotinylated swine anti-rabbit IgG and rabbit anti-mouse IgG antibodies were then applied, and a streptavidin-peroxidase conjugate was finally used for visualisation with DAB (3,3′-diaminobenzidine tetrahydrochloride) as the final acceptor. Appropriate controls were used.

A modified Timm sulphide-silver method (SSM) was used to demonstrate iron cytochemically as previously described.[22] Briefly, deparaffined sections (5 μm thick) of arteries were initially re-fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate–HCl buffer with 0.1 M sucrose, pH 7.2 (total osmolality 510 and effective osmolality 300 mOsm) for 1–2 h. Following a short rinse in double-distilled water, the specimens were sulfidated in 1% ammonium sulphide in 70% ethanol for 15 min and again carefully rinsed with distilled water. The sections were then developed in a physical developer containing Ag-lactate (0.11 g in 15 ml distilled water), hydroquinone (0.85 g in 15 ml distilled water), 60 ml 25% gum arabic, and 10 ml Na-citrate buffer (pH 3.8) for 15–30 min. For light microscopy, the sections, with or without a light nuclear counterstaining, were mounted in Canada balsam after dehydration in a gradient series of ethanol. SSM is a very sensitive cytochemical technique, by forming a shell of silver around a core of MeS which is formed by exposing tissues to H_2S or $(NH_4)_2S$. Only iron which is not firmly bound in stable metallo-organic compounds is converted to FeS (and thus later demonstrated by the development procedure). The type of iron that becomes converted to FeS is thus of low-molecular weight. Since the exposure to deferoxamine considerably reduced such iron deposition in Fe-exposed cells,[22] it is reasonable to assume that this method may mainly detect low-molecular-weight iron.

3. Detection of Apoptotic Cells

Apoptotic cells in the artery walls were shown using the TUNEL technique (*in situ* *T*erminal deoxynucleotidyl transferase-mediated d*U*TP *N*ick *e*nd *L*abelling), according to the manufacturer's instructions (Apoptag®, Oncor, Gaithersburg, MD, USA). In brief, paraffin sections of arterial walls were deparaffinised in xylene, rehydrated, incubated with proteinase K, and washed with 2% H_2O_2. Sections exposed to DNase were positive controls. After a wash in an equilibration buffer, the sections were further incubated with the TdT (*T*erminal *d*eoxynucleotidyl *T*ransferase) enzyme for 1 h at 37°C, and with an anti-digoxigenin-peroxidase complex for another 30 min at room temperature. Negative controls were run without the TdT enzyme. Counter staining was done with methylgreen for 30 s.

4. Cell Counting and Image Analysis

For the evaluation of TUNEL, ferritin, and CD68 stainings, 500 cells were counted in the intima, media, and adventitia of each specimen, respectively. The percentages of positive cells in the three layers were calculated.

To compare the relation of positive areas and case distribution, scoring criteria were used to examine all the specimens. The scores refer to: 0, as negative; 1, as a few positive cells without

cluster formation; 2, as positive areas less than 1/4 field of vision (×20); 3, positive areas larger than 1/4 field of vision (×20).

The immunohistochemical and TUNEL reactions were analysed with computerised image analysis. Five images of intima, media, and adventitia of each specimen were randomly obtained from representative areas using a Leitz DM-RBE microscope equipped with a HAMATSU CCD video camera, and connected to a Macintosh desktop computer (8100/110). The acquired colour micrographs (412552 pixels) were transferred and assessed using an Adobe Photoshop (Adobe System Inc., USA).[23] The size of the stained areas was presented as number of pixels.

5. Statistics

The Wilcoxon signed rank and Mann–Whitney U tests were used to analyse differences between atherosclerotic lesions and control arteries from the forensic and the clinical cases. A probability value of less than 5% ($p < 0.05$) was considered to be significant.

RESULTS

1. Comparison of Ferritin Accumulation and Apoptosis in CD68 Positive Macrophages

Table I is a summary of the major histopathological findings of all autopsy cases (12 cases with general atherosclerosis and 7 young forensic cases without atherosclerosis). No CD68- or ferritin positive cells were found in the normal coronary arteries from the young forensic cases, while a certain number of ferritin-positive cells were found in small clusters of CD68-positive cells from normal appearing parts of arteries from the atherosclerosis cases. Pronounced ferritin accumulation and apoptosis were generally the findings in many of the CD68-positive macrophages that were found in the atherosclerotic lesions of the diseased cases (Figures 1 and 2). A few

TABLE I Data on autopsy cases

Age/Sex	Artery	CD68	Ferritin	TUNEL	Cause of death
Young individuals without atherosclerosis					
9/F	Coronary	–	–	+	Accident
27/M	Coronary	–	–	+	Accident
30/F	Coronary	–	–	–	Accident
10/F	Coronary	–	–	+	Accident
21/F	Coronary	–	–	+	Accident
23/M	Coronary	–	–	+	Suicide
22/M	Coronary	–	–	+	Accident
Old individuals with atherosclerosis					
45/F	Aorta	+	+	NA	GA
73/M	Aorta	–	+	NA	GA
82/M	Aorta	+	+	NA	GA
40/M	Aorta	+	+	NA	GA
75/M	Coronary	+	+	NA	GA
84/F	Coronary	+	+	+	GA
88/M	Coronary	+	+	+	GA
88/F	Coronary	+	+	+	GA
68/M	Coronary	–	+	+	GA
64/F	Coronary	–	–	+	GA
63/M	Coronary	+	+	+	GA
80/M	Coronary	+	+	+	GA

F, female; M, male; GA, general atherosclerosis; NA, not applied; –, negative; +, positive.

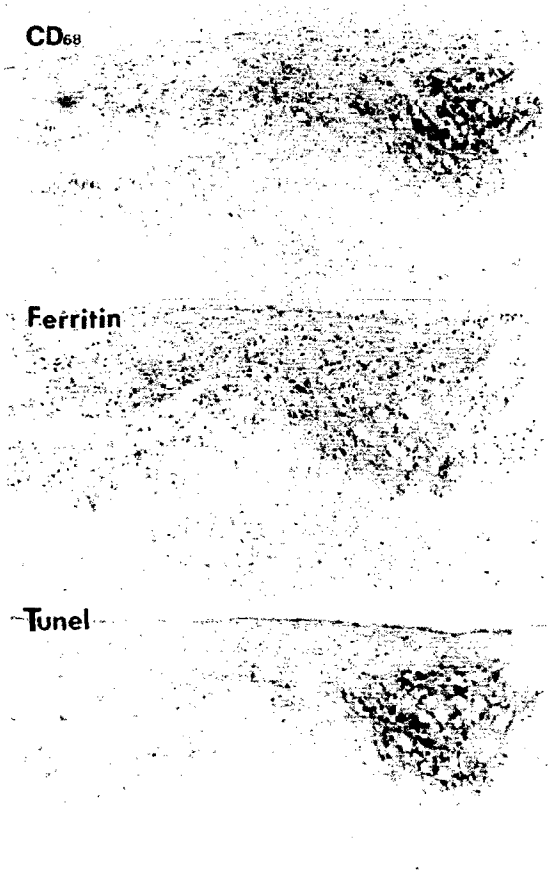


FIGURE 1 CD68- and ferritin-immunohistochemistry and TUNEL-positivity of an atherosclerotic lesion. Note combined positivity within the same area of a micro-atheroma (adjacent sections, ×100). (See Color plate I at the end of this issue.)

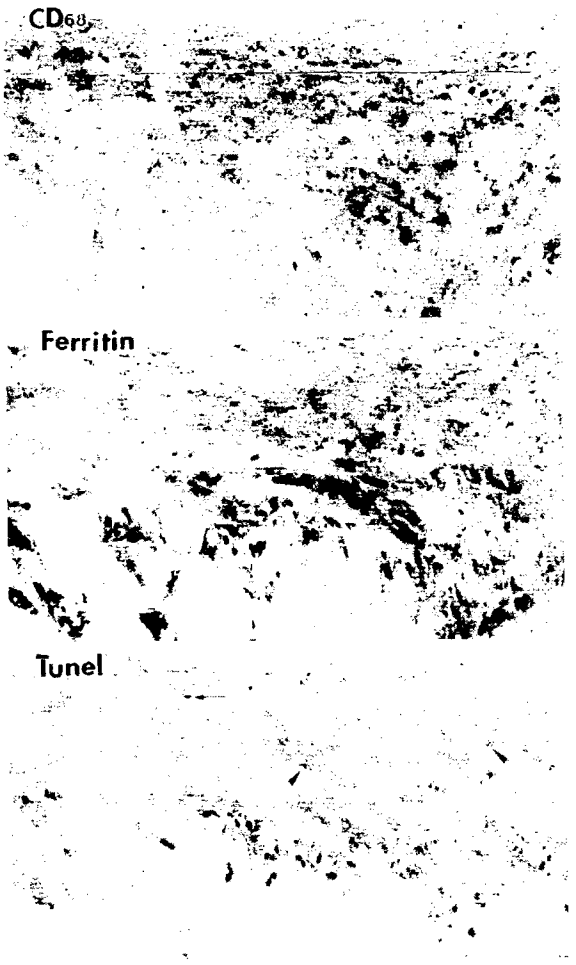
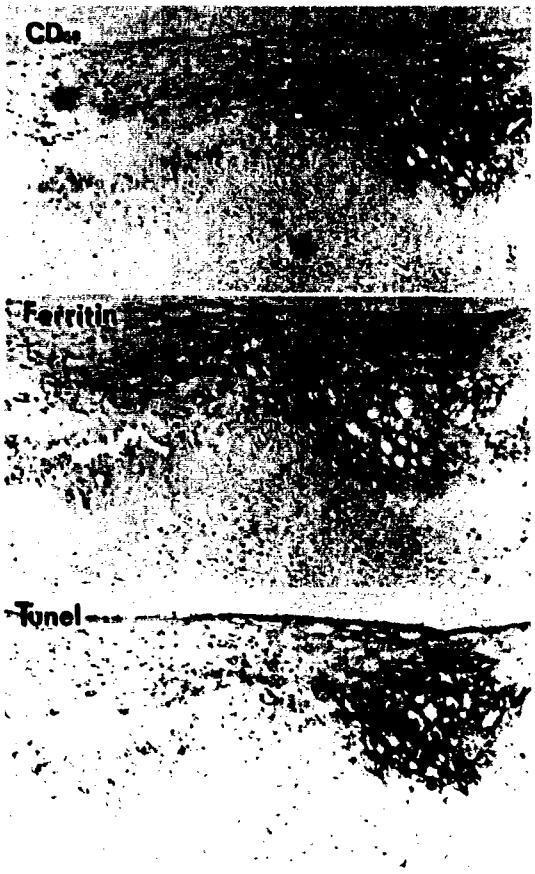


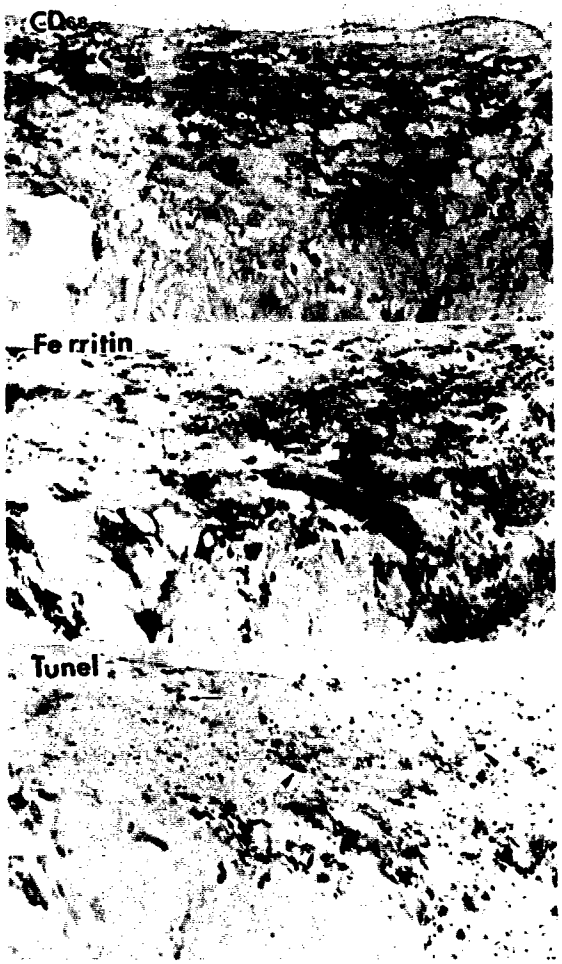
FIGURE 2 CD68- and ferritin-immunohistochemistry and TUNEL-positivity of the shoulder region in an atherosclerotic lesion. High-power view of ferritin accumulation and apoptosis in CD68-positive macrophages and foam cells. The CD68-, ferritin- and TUNEL-positivity are localised in the same area. Arrowheads and arrows indicate nuclear- or cytosolic positivity of TUNEL, respectively (×400). (See Color plate II at the end of this issue.)

apoptotic cells were present in the normal coronary arteries from some of the forensic cases (Figure 3).

According to the scoring criteria, all the sections stained for CD68, ferritin, and apoptosis (TUNEL) were repeatedly examined, at least three times, and the results summarised in Figure 3. Compared with normal appearing parts of the atherosclerotic arteries, significantly higher amounts of ferritin were found in CD68 positive cells of atheroma tissues. Even though only normal-appearing parts of atherosclerotic arteries were selected for use as one of the controls, some ferritin- and CD68-positive cells, occasionally forming minute clusters in the intima, were found. This finding is probably an indication of early atheroma formation which was still not macroscopically evident. Such material, thus, could not be used as true controls. As another type of control we also used normal coronary arteries (here called normal arterial tissue) from young forensic cases. In these tissues we did not find ferritin or CD68-positive cells, although a few apoptotic ones.



Color Plate I (see page 225, figure 1) CD68- and ferritin-immunohistochemistry and TUNEL-positivity of an atherosclerotic lesion. Note combined positivity within the same area of a micro-atheroma (adjacent sections, ×100).



Color Plate II (see page 225, figure 2) CD68- and ferritin-immunohistochemistry and TUNEL-positivity of the shoulder region in an atherosclerotic lesion. High-power view of ferritin accumulation and apoptosis in CD68-positive macrophages and foam cells. The CD68-, ferritin- and TUNEL-positivity are localised in the same area. Arrowheads and arrows indicate nuclear- or cytosolic positivity of TUNEL, respectively (×400).

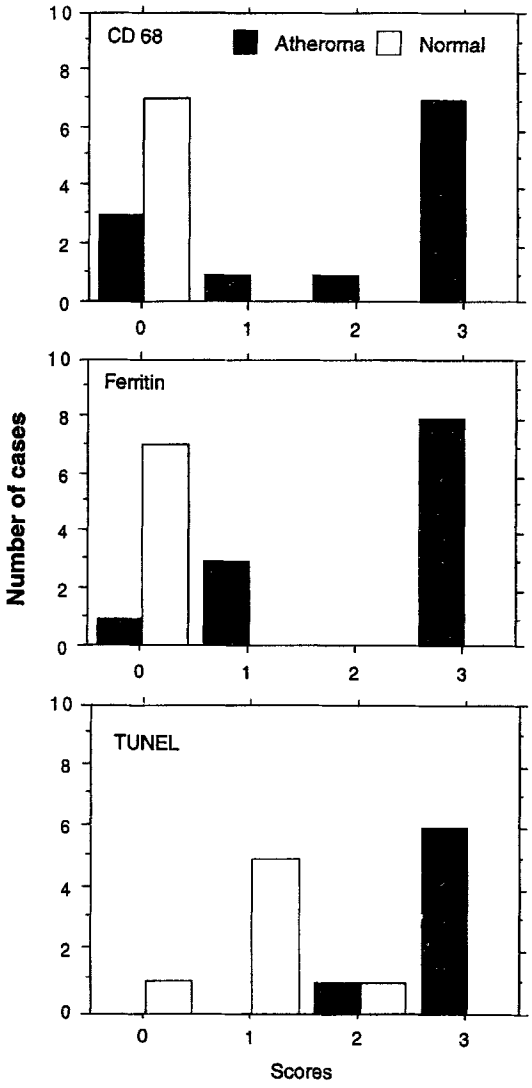


FIGURE 3 Scorings for CD68-, ferritin-immunocytochemistry, and positive TUNEL reaction in normal arteries from young forensic cases and atherosclerotic vessels from the clinical cases. Ferritin accumulation in CD68-positive cells were found in most of the atherosclerotic lesions with the highest scoring. No CD68- and ferritin-positive cells were found in the normal arteries of the forensic cases, although some apoptotic cells were detected.

2. Distribution of Iron/Ferritin Laden Macrophages

Atherosclerotic vessels from eight of the twelve clinical cases showed strong positivity for ferritin (score >2, see Figure 3); only one case was negative. No ferritin was found in arteries from the young individuals' coronary arteries. The scores of CD68-, ferritin- and TUNEL-stainings showed strikingly different distributions between normal arterial tissues and atheromas (Table I and Figure 1). In atherosclerotic lesions, ferritin was predominantly present in the subendothelial intimal space and at the outer edge of the lipid core and shoulder regions with a high density of macrophages. These findings correspond well with our previous results on iron location in atheromas using the sensitive SSM.[5]

By applying the anti-CD68 antibodies to serial sections, we noticed areas of ferritin-positive macrophages to be frequently mixed with erythrocytes (Figure 4). This finding may give support to our suggestion that erythrophagocytosis due to microbleedings may be a way for macrophages to take up iron and subsequently mediate LDL-oxidation by exocytosing this transition metal.[4] Figure 5 shows that ferritin-positive cells, mainly macrophages, are also rich in low-molecular-weight iron as proved by the SSM. Pronounced TUNEL-positivity was also found in the same areas, indicating that low-molecular-weight iron may be involved in the apoptotic process of macrophages and foam cells.

3. Distribution of Apoptotic Macrophages

Atherosclerotic lesions from the clinical cases, and normal arterial tissues from the young forensic cases, were analysed and compared for the presence of apoptosis (see Table II). A substantial number of apoptotic cells were seen in all examined atherosclerotic lesions compared with normal arteries which contained only a few such cells ($p < 0.001$). Interestingly, in normal arterial tissues, a few TUNEL-positive cells appeared mainly in the subendothelial zone containing CD68-positive macrophages. This may indicate that macrophages actively participate in the low physiological level of apoptosis in normal vessels,

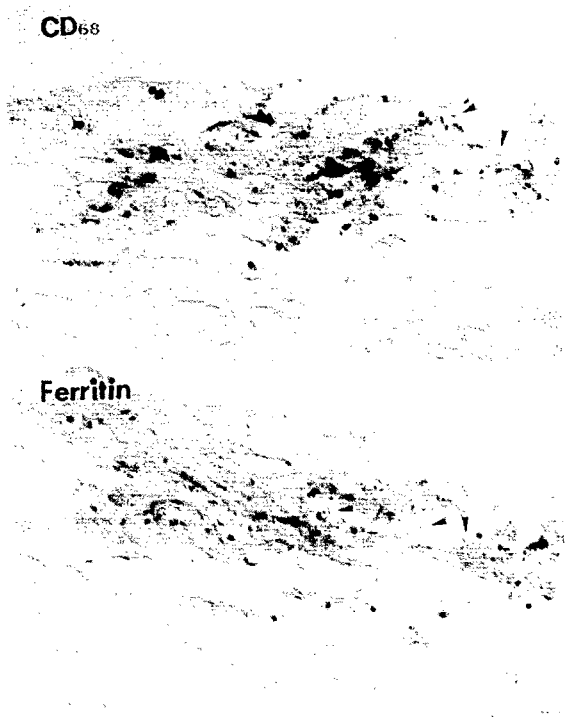


FIGURE 4 CD68- and ferritin-immunohistochemistry of an atherosclerotic lesion by transmitted-light-interference-contrast-microscopy. CD68-positive areas are also positive with respect to ferritin (on adjacent sections). Note the presence of erythrocytes arrowed surrounding the macrophages (×400). (See Color plate III at the end of this issue.)

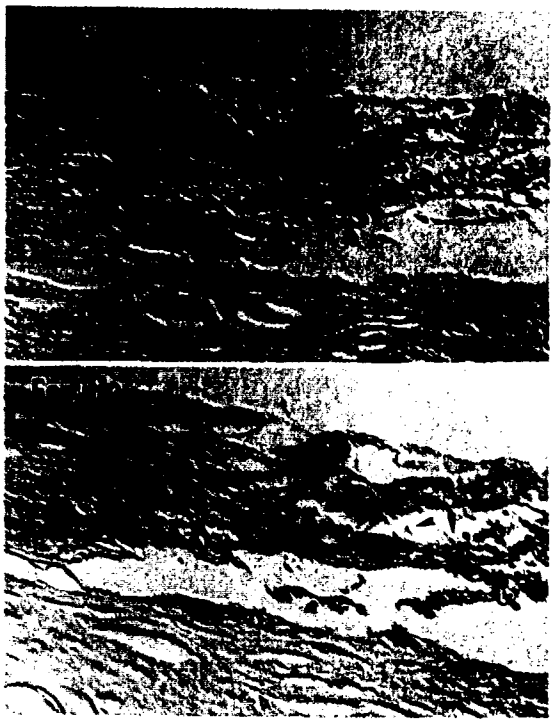
FIGURE 5 Ferritin immunohistochemistry (A), SSM-iron histochemistry (B), and TUNEL reaction (C) in an atheroma. Ferritin-positive cells, mainly macrophages rich in lipids, show ongoing apoptosis, and are also positive in the SSM staining (×400). (See Color plate IV at the end of this issue.)

which is associated with the migration and recruitment of monocyte-derived macrophages. We found prominent apoptosis in the macrophage-enriched shoulder area surrounding the lipid core of the atheromas, though some apoptotic cells were seen throughout the vessel wall. There were two types of TUNEL-positive macrophages: truly apoptotic cells with nuclear-positivity due to inter-nucleosomal DNA fragmentation; and cells with cytoplasmic positivity due to the phagocytosis of apoptotic bodies. The difference between the two types of TUNEL-positive cells is shown in Figure 6. There was a significant difference between atheroma and normal arterial tissues. Cells with cytoplasmic TUNEL-positive fragments were mainly found in the intima and adventitia of atherosclerotic

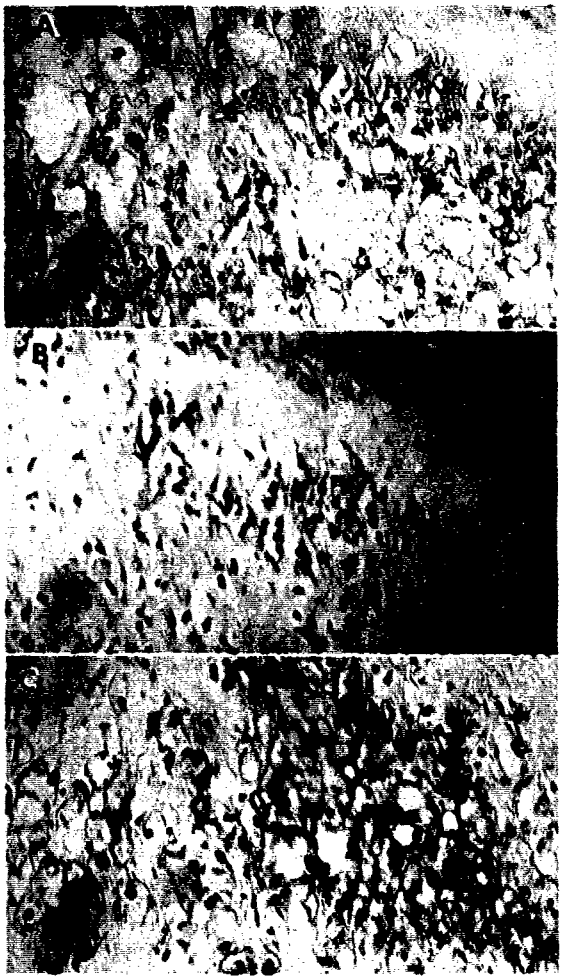
TABLE II TUNEL positive cells in different layers of coronary arteries

	Intima		Media		Adventitia	
	NP (%)	CP (%)	NP (%)	CP (%)	NP (%)	CP (%)
Atheroma	22.0	2.9	19.9	0.6	23.2	3.7
Normal vessels	4.9	0.3	4.8	0.8	7.6	1.7

NP, Nuclear positivity; CP, Cytoplasmic positivity ($n = 7$). In each layer 500 cells were counted.



Color Plate III (see page 227, figure 4) CD68- and ferritin-immunohistochemistry of an atherosclerotic lesion by transmitted-light-interference-contrast-microscopy. CD68-positive areas are also positive with respect to ferritin (on adjacent sections). Note the presence of erythrocytes arrowed surrounding the macrophages (×400).



Color Plate IV (see page 227, figure 5) Ferritin immunohistochemistry (A), SSM-iron histochemistry (B), and TUNEL reaction (C) in an atheroma. Ferritin-positive cells, mainly macrophages rich in lipids, show ongoing apoptosis, and are also positive in the SSM staining (×400).

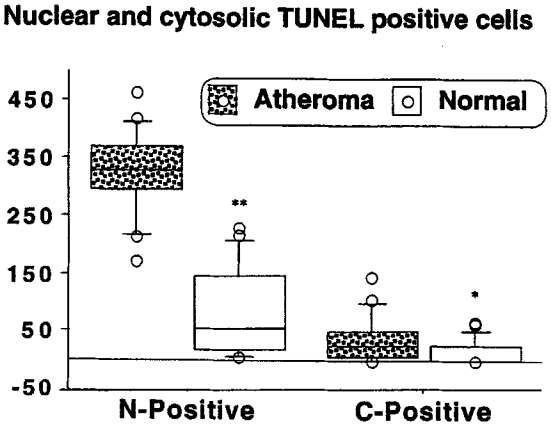


FIGURE 6 TUNEL-positive cells in normal arteries from the young forensic cases and in atherosclerotic vessels from the clinical cases. The box blot graph shows significant differences in nuclear- and cytosolic-positive TUNEL reactions between normal and atheroma samples ($^{*}p < 0.05$; $^{**}p < 0.001$) N-positive: nuclear positivity; C-positive: cytosolic positivity.

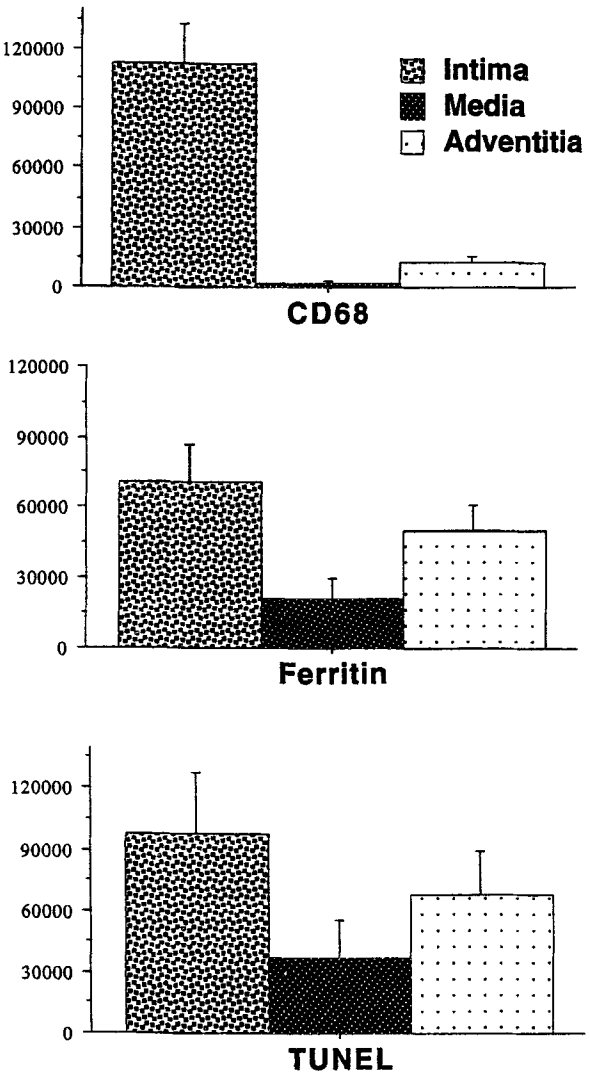


FIGURE 7 Image analyses of CD68- and ferritin-immunohistochemistry and TUNEL-positivity of atherosclerotic vessels. The pixel values of the three stainings were uniformly high in the atherosclerotic intima, while this was not the case for the media and adventitia.

vessels. Extracellular TUNEL-positive fragments were often found in regions with dead cells. Surprisingly, the adventitia of atherosclerotic arteries contained a rather high percentage of apoptotic cells and inflammatory cells. Also, it was noticed that TUNEL-positive smooth muscle cells were present in the media of atherosclerotic vessels.

The coexistence of CD68-, ferritin-, low-molecular-weight iron, and TUNEL-positivity within the same atheromatous area was obvious. The result of the image analyses is summarised in Figure 7. In atherosclerotic intimas there was almost the same number of CD68-, ferritin- and TUNEL-pixels, while in the media and adventitia the ferritin- and TUNEL-pixels reached the same levels, although the one for CD68 was lower. This indicates that macrophages play a dominating role in ferritin accumulation and apoptosis within the atheromatous intima, while in other parts of the atheroma ferritin accumulation and apoptosis could be associated also with other types of cells.

DISCUSSION

In atherosclerotic lesions there is a certain amount of redox-active iron, mainly in macrophages and macrophage-derived foam cells.[5,24] In this study we found that these cells are also rich in ferritin and there is a marked difference between atheromas and normal arterial tissues with respect to

the frequency of apoptotic- and ferritin-rich macrophages. Apoptosis frequently occurs in atherosclerotic and, to some degree, also in normal tissues, indicating that programmed cell death is a normal process within arterial walls, although it may be abnormally activated during the progression of atherosclerosis. A major finding of this study was the association between prominent apoptosis and demonstrable iron/ferritin within macrophages/macrophage-derived foam cells in human lesions, suggesting iron-catalysed reactions to be involved in apoptosis and plaque instability.

Ferritin, the major iron-storing protein, is considered to be an antioxidant since it sequesters intracellular iron (mainly by the L-chain) and also has ferroxidase capacity (by the H-chain), keeping iron in ferric form without the capacity to induce Fenton-reaction. On the other hand, ferritin has been reported to release redox-active iron under certain conditions and, thus, to be a prooxidant as well.[11] A recent study indicates that iron still bound to ferritin, rather than released, is likely responsible for catalytic action resulting in cytotoxicity.[25] The finding of abnormally increased amounts of ferritin, and its increased gene expression in human atherosclerotic lesions,[11] prompted us to consider the pathological implications of such excessive ferritin accumulation in the progression of atherosclerosis. The relative absence of CD68- and ferritin-positivity in normal arterial tissues indicates that abnormal sequestration of ferritin within macrophages and macrophage-derived foam cells within atherosclerotic lesions would either result from, or be the cause of, the atherosclerotic process. The detailed pathways and mechanisms need to be further clarified.

The source of free iron, the cause of ferritin accumulation, and their relation to LDL oxidation and atheroma progression, are crucial but unanswered questions. Accumulating evidence suggests that iron, haeme, and ferritin may initiate oxidative stress and enhance LDL oxidation. Small amounts of haemoglobin, a major iron-containing protein in erythrocytes, were reported to trigger LDL apoB-100 crosslinking and peroxidative oxidation.[26] Based on our studies we hypothesise that the degradation of aged erythrocytes, or apoptotic cells, is a major way for macrophages to sequestrate iron, initially within lysosomes, and later in the form of cytosolic ferritin. Iron may be also released to the extracellular environment by exocytosis. In this study, the shown association of ferritin-rich macrophages with microbleeding areas may shed some light on the mechanisms behind iron-sequestration in macrophages of human atheromas. However, the more direct association between free haeme, or hemoglobin, and LDL oxidation *in vivo* merits further investigations.

Apoptosis in atherosclerotic lesions has been described in man and animal using the TUNEL technique. Because of the unavoidable overestimation of apoptosis by TUNEL, as every living cell has a few single strand breaks (80–100,000 base pairs per cell) under repair,[17,27] and different criteria for the quantification of the reaction product are applied, controversial frequencies of apoptosis in atherosclerotic lesions and control tissues have been presented. However, since other approaches for the examination of apoptosis are not applicable for *in situ* examination of overall artery tissues, we still consider that the TUNEL technique is the most suitable way to investigate apoptosis in atherosclerotic plaques. Based on observations with this technique, most authors agree that the number of apoptotic cells is highest in regions enriched with macrophages/macrophage-derived foam cells, and, moreover, that apoptosis in atheroma tissues could be an important event during the development of atherosclerosis. Free radicals and transition metals have been proposed to play important roles in the induction and mediation of apoptosis, although more evidence is required to establish this notion.[28] To our knowledge, the present study is the first report on a correlation between iron and apoptosis in human atherosclerotic lesions.

Studies of atherosclerotic vessels have shown that the most vulnerable area is at the shoulders

of atheromas. Particularly, macrophage-rich areas of atherosclerotic lesions has been considered a marker of unstable atherosclerotic plaques.[29] The coexistence of ferritin and apoptosis within macrophage/foam cells at the atheroma shoulders is a new challenge for the understanding of progression. Although apoferritin is also enriched in early atherosclerotic lesions, without any major iron deposits as assayed by the Pearl's histochemical technique for iron,[7] it is yet too early to conclude that ferritin-accumulation in atherosclerotic plaques is a cytoprotective event. Two recent studies from our group showed that induction of ferritin synthesis is not always enough to protect the cells from oxidative stress.[30,31] We speculate that, at least in advanced atheromas, macrophages overloaded with ferritin-bound iron, following uptake of aged erythrocytes or damaged inflammatory cells, may show enhanced sensitivity to oxidative stress, resulting in apoptosis. Even if newly synthesised apoferritin certainly could bind free iron, and thus protect against harmful iron-catalysed oxidative reactions, this seems to be an inefficient protection. The reason may be that autophagocytosed ferritin would be degraded and release low-molecular-weight iron intralysosomally with consequent enhanced lysosomal sensitivity to oxidative stress.[30,31] Both ferritin accumulation and apoptosis occur more often in the atheroma shoulder regions, indicating that excessive iron-sequestration in ferritin may be linked to instability and rupture of atherosclerotic plaques.

In conclusions, low-molecular iron and ferritin both accumulates in macrophages and macrophage-derived foam cells of human atherosclerotic lesions. Dysregulated iron- and ferritin-metabolism within macrophage/foam cells is suggested to be associated with ongoing apoptosis contributing to the instability of atherosclerotic plaques. The relation between ferritin-sequestration and apoptosis of macrophages in atheromas, and the significance of accumulated ferritin for the progression of atherosclerosis requires further investigations.

Acknowledgements

This work was supported by the Swedish Medical Research Council (grants 4481 and 6962). The author wishes to express his gratitude to Profs. Ulf T. Brunk and Anders G. Olsson for their valuable suggestions and critical reading of the manuscript. The author also thanks Ms Birgitta Frånlund and Ms Tina Anderson for excellent technical assistance.

References

[1] Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L. (1989) Beyond cholesterol: modifications of LDL that increase its atherogencity. *New England Journal Medicine*, **320**, 915–924.
[2] Witztum, J.L. and Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherogenesis. *Journal of Clinical Investigation*, **88**, 1785–1792.
[3] Halliwell, B. and Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymol*, **186**, 1–85.
[4] Yuan, X.M. and Brunk, U.T. (1998) Iron and LDL-oxidation in atherogenesis. *APMIS*, **106**, 825–842.
[5] Yuan, X.M., Li, W., Olsson, A.G. and Brunk, U.T. (1996) Iron in human atheroma and LDL oxidation by macrophages following erythrophagocytosis. *Atherosclerosis*, **124**, 61–73.
[6] Yuan, X.M., Olsson, A.G. and Brunk, U.T. (1996) Macrophage erythrophagocytosis and iron exocytosis. *Redox Report*, **2**, 9–17.
[7] Juckett, M.B., Balla, J., Balla, G., Jessurun, J., Jacob, H.S. and Vercellotti, G.M. (1995) Ferritin protects endothelial cells from oxidized low density lipoprotein *in vitro*. *American Journal Pathology*, **147**, 782–789.
[8] Kiech, S., Willeit, J., Egger, G., Poewe, W. and Oberhollenzer, F. (1997) Body iron stores and the risk of carotid atherosclerosis. Prospective results from the Bruneck study. *Circulation*, **96**, 3300–3307.
[9] Kiechl, S., Aichner, F., Gerstenbrand, F., Egger, G., Mair, A., Rungger, G., Spogler, F., Jarosch, E., Oberhollenzer, F. and Willeit, J. (1994) Body iron stores and presence of carotid atherosclerosis. Result from the Bruneck study. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **14**, 1625–1630.
[10] Salonen, J.T., Nyyssönen, K., Loral, H., Tuomilehto, J., Seppanen, R. and Salonen, R. (1992) High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. *Circulation*, **86**, 803–811.
[11] Reif, D. (1992) Ferritin as a source of iron for oxidative damage. *Free Radical Biology Medicine*, **12**, 417–427.
[12] Dai, L., Winyard, P.G., Zhang, Z., Blake, D.R. and Morris, C.J. (1996) Ascorbate promote low density lipoprotein oxidation in the presence of ferritin *Biochimca et Biophysica Acta*, **1304**, 223–228.
[13] Pang, J.H., Jiang, M.J., Chen, Y.L., Wang, F.W., Wang, D.L. and Chu, S.H. (1996) Increased ferritin gene expression in atherosclerotic lesions. *Journal of Clinical Investigation*, **97**, 2204–2212.

[14] Isner, J.M., Kearney, M., Bortman, S. and Passeri, J. (1995) Apoptosis in human atherosclerosis and restenosis. *Circulation*, **91**, 2703–2711.
[15] Han, D.K.M., Haudenschild, C.C., Hong, M.K., Tinkle, B.T., Leon, M.B. and Liau, G. (1995) Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. *American Journal Pathology*, **147**, 267–277.
[16] Geng, Y.J. and Libby, P. (1995) Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 β-converting enzyme. *American Journal Pathology*, **147**, 251–266.
[17] Kockx, M.M., De Meyer, G.R.Y., Muhring, J., Bult, H., Bultinck, J. and Herman, A.G. (1996) Distribution of cell replication and apoptosis in atherosclerotic plaques of cholesterol-fed rabbits. *Atherosclerosis*, **120**, 115–124.
[18] Hegyi, L., Skepper, J.N., Cary, N.R.B. and Mitchinson, M.J. (1996) Foam cell apoptosis and the development of the lipid core of human atherosclerosis. *Journal of Pathology*, **180**, 423–429.
[19] Yuan, X.M., Li, W., Olsson, A.G. and Brunk, U.T. (1997) The toxicity to macrophages of oxidized low-density lipoprotein is mediated through lysosomal damage. *Atherosclerosis*, **133**, 153–161.
[20] Li, W., Yuan, X.M., Olsson, A.G. and Brunk, U.T. (1998) Uptake of oxidized LDL by macrophages results in partial lysosomal enzyme inactivation and relocation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **18**, 177–184.
[21] Stary, H.C., Chandler, B., Robert, E. *et al.* (1995) A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the committee on vascular lesions of the council on arteriosclerosis, American Heart Association. *Arteriosclerosis, Thrombosis, and Vascular Biology*, **15**, 1512–1531.
[22] Zdolsek, J., Roberg, K. and Brunk, U.T. (1993) Visulization of iron in cultured macrophages: a cytochemical light and electron microscopic study using autometallography. *Free Radical Biology Medicine*, **15**, 1–11.
[23] Sittinger, M., Burmester, G. and Bujia, J. (1994) Inexpensive computer analysis of cell and tissue stainings. *In Vitro Cellular Developmental Biology*, **30A**, 559–561.
[24] Smith, C.A., Mitchinson, M.J., Aruoma, O.I. and Halliwell, B. (1992) Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochemical Journal*, **286**, 901–905.
[25] Roginsky, V., Barsukova, T.K., Bruchelt, G. and Stegmann, H.B. (1997) Iron bound to ferritin catalyzes ascorbate oxidation: effects of chelating agents. *Biochimca et Biophysica Acta*, **1335**, 33–39.
[26] Miller, Y.I., Felikman, Y. and Shaklai, N. (1996) Hemoglobin induced apolipoprotein B crosslinking in low-density lipoprotein peroxidation *Archives of Biochemistry and Biophysics*, **326**, 2252–2262.
[27] Gold, R., Schmied, M., Giegerich, G., Breitschoph, H., Hartung, H.P., Toyka, K.V. and Lassmann, (1994) Differentiation between cellular apoptosis and necrosis by the combined use of the *in situ* tailing and nick translation techniques. *Laboratoray Investigation*, **71**, 219–225.
[28] Wolfe, J., Ross, D. and Cohen, G.M. (1994) A role of metals and free radicals in the induction of apoptosis in thymocytes. *FEBS Letters*, **352**,58–62.
[29] Davies, M. (1996) Stability and instability. Two faces of coronary atherosclerosis. The Paul Dudley White Lecture 1995. *Circulation*, 2013–2020.
[30] Garner, B., Li, W., Roberg, K. and Brunk, U.T. (1997) On the cytoprotective role of ferritin in macrophages and its ability to enhance lysosomal stability. *Free Radical Research*, **27**, 487–500.
[31] Garner, B., Roberg, K. and Brunk, U.T. (1998) Endogenous ferritin protects cells with iron-laden lysosomes againt oxidative stress. *Free Radical Research*, **29**, 103–114.