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Erythrophagocytosis and Iron Deposition in Atherosclerotic Lesions

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Abstract

Iron deposition has been shown to be prominent in atherosclerotic lesions. However, the source of iron accumulated in arterial walls is unclear. In present report, we provide the histological evidence to demonstrate the localization of erythrocytes in atherosclerotic lesions from experimental animals. As revealed by scanning and transmission electron microscopy, the circulating erythrocytes were found to be present in intima of atherosclerotic aortas from apoE-deficient mice. These erythrocytes appeared to be readily phagocytosed by macrophages in lesions. The erythrophagocytosis was also evident in lesions from cholesterol-fed rabbits. Furthermore, the iron deposition was detectable in the region with erythrocytes. When the aortic sections of humans and apoE-deficient mice were immunostained with specific antibody to hemoglobin, it was clearly shown that the positive stain was detectable in macrophage-derived foam cells. Immunostaining of serial sections with specific antibodies to heme oxygenase-1 (HO-1) and ferritin further demonstrated the colocalization of HO-1 and ferritin in area with positive immunoreactivity for hemoglobin. Likewise, Perls' reaction revealed the positive iron stain in the same region. Collectively, these results suggest that hemoglobin/heme released from the phagocytosed erythrocytes may contribute to at least part of iron deposited in atherosclerotic lesions.

Key Words: erythrophagocytosis, iron, atherosclerosis

Introduction

Atherosclerosis is a disease with complicated etiology. Although the pathogenesis of this disease is not yet fully understood, histological assessment has revealed that the infiltration of circulating monocytes and T lymphocytes in aortic subendothelial intima occur in the early phase of atheroclerosis (1). The monocytes then undergo differentiation to macrophages and uptake the oxidized low-density lipoprotein (LDL) to become foam cells, which are the characteristic hallmark of early lesions. Although erythrocytes account for over 95% of the blood cells in circulation, the localization of erythrocytes in atherosclerotic lesions of early stage was rarely demonstrated.

It is well known that the main function of erythrocytes, which carry about 65% of the total quantity of iron in the body by hemoglobin, is to deliver oxygen to all tissues and cells. Nevertheless, it has been speculated that the hemoglobin can be the potential source of noxious free iron to damage the vasculature via Fenton reaction to produce the reactive oxygen species (2). This notion was supported by previous studies showing that hemoglobin or free heme could potently sensitize endothelium to oxidant -mediated cytotoxicity (3, 4). The oxidative potential of hemoglobin/heme was also demonstrated by its ability to catalyze the oxidation of LDL (5-7), which is one of the crucial events in lesion development. In either event, the hemoglobin/heme released from disrupted erythrocytes can be atherogenic.

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Recently, evidence was accumulating to show that the redox active iron is present in human atherosclerotic plaques (8, 9). Studies from our laboratory also revealed that iron deposition is prevalent in human advanced lesions (10, 11). Nevertheless, the source of iron accumulated in vascular wall remains to be clarified. Since the erythrocytes in circulation can be the potential source of iron, we are interested in exploring the possibility whether erythrocytes are present in atherosclerotic arterial walls. In present study, we provide the histological evidence to demonstrate the erythrophagocytosis by cells in lesions of intermediate stage from experimental animals. The colocalization of immunoreactivities for hemoglobin, heme oxygenase −1 (HO-1), and ferritin in area rich in macrophages suggests that the iron derived from hemoglobin may account for, at least in part, the iron deposited in atherosclerotic lesions.

Materials and Methods

Human samples. Human ascending aortas (n=3) were obtained from patients during bypass surgery for ischemic heart disease. The sampling of patient specimens followed the procedure of the hospital's ethical committee, and the study approved by the human subject review committee in our institute. Immediately after surgery, tissue samples were rinsed with ice-cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde solution, and paraffin-embedded.

Animals

The homozygous apoE-deficient mice in C57BL/6J background were purchased from the Jackson Laboratory and maintained under conventional housing conditions in our animal facility. These mice (n=5) were placed on chow diet and sacrificed at ages of 5-7 month old. Homozygous Wanatabe heritable hyperlipidemic (WHHL) rabbits were generously supplied by Dr. Masashi Shiomi, Kobe University, Japan. Heterozygous WHHL rabbits were generated by crossing the homozygous WHHL rabbits with New Zealand white rabbits. The 6 month old WHHL heterozygotes (n=3) were fed with chow diet supplemented with 0.5% cholesterol for 2 months prior to sacrifice. Aortic tissues obtained from animals were proceeded as described above for human samples. For electron microscopy, tissues were fixed in 2.5% glutaraldehyde in 100 mM Na-phosphate buffer overnight, washed with the same buffer for 15 min, and postfixed with 1% osminm tetroxide in 100 mM phosphate buffer at room temperature for 2 hrs.

Electron microscopy

For scanning electron microscopy, samples were dehydrated with graded series of ethanol (50-100%) and then critical-point dried in CO 2. After mounted on aluminum stubs and coated with palladium-gold, samples were examined with JSM-T330A scanning electron microscope. For transmission electron microscopy, samples were dehydrated with graded series of ethanol (50-100%) and then embeded in Spurr. The thin sections of 80 nm were prepared using ultramicrotome and placed on 200 or 300 mesh copper grids. Sections were stained with uranyl acetate and lead citrate and examined with a JEM 1200 EX transmission electron microscope at 80 kv.

Hematoxylin and Eosin Y staining

Paraffin-embedded sections were stained with Mayer's hematoxylin solution for 3 min, washed with tap water for 15 min and stained with eosin Y for another 5 min. After dehydration with increasing concentrations of ethanol from 70% to 100% followed by immersion into xylene for 5 min three times, slides were mounted and examined by light microscope.

Immunohistochemistry

Immunostaining was carried out using the following antibodies: rabbit anti-human hemoglobin polyclonal antibody (Dako, Kyoto, Japan), mouse anti-human macrophage CD68 monoclonal antibody (Dako), mouse anti-human smooth muscle cell α-actin monoclonal antibody (Dako), rabbit anti-human ferritin polyclonal antibody (Dako), rabbit anti-rat HO-1 polyclonal antibody (StressGen Biotechnologies, Victoria, Canada) and rat anti-mouse macrophage F4/80 monoclonal antibody (Serotec, Kidlington, England). Paraffin sections (5 μm) were rehydrated and washed with PBS. Endogenous peroxidase activity was exhausted by incubation with 3% H_2O_2 for 10 min at room temperature. Sections were then incubated with PBS containing 1% goat serum albumin and 1% bovine serum albumin for 30 min at 37°C. After 3 washes with PBS, sections were incubated with first antibody in PBS for 30 min at 37°C. After washing, sections were incubated with peroxidase-conjugated secondary antibodies for another 30 min at 37°C. Color reactions were then developed by incubation with 0.1% 3,3'-diaminobenzidine (DAB). Negative controls were carried out by omission of the first antibody or substitution of normal rabbit IgG for the first antibody.

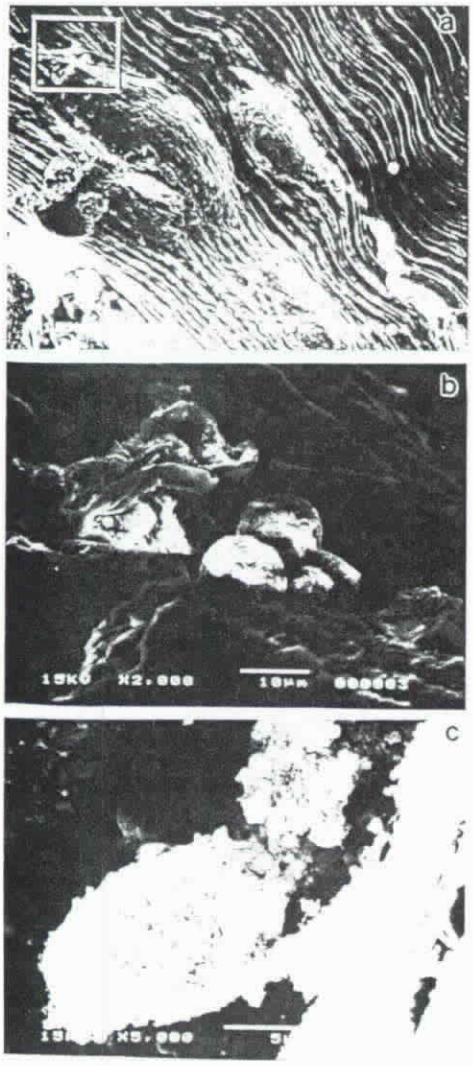

Fig. 1. Scanning electron microscopy of the atherosclerotic aorta from a 6 month old apoE-deficient mouse. Higher power view of the framed area in (a) was shown in (b). Magnification, X200 (a), X2,000 (b), X5,000 (c).

Iron Histochemistry

Iron deposition was examined by Perls' Prussian blue reaction as described previously (10). For intensifying the signal, after Perls' reaction, sections were incubated with 0.5% DAB in 100 mM phosphate buffer pH7.4 for 20 min, followed by 15 min in the same medium containing 0.01% H_2O_2. The reaction was stopped by rinsing in deionized H_2O for 30 min and then counterstained with carmin red. Negative control was carried out through the DAB intensification without preincubation with Perls' solution.

Results

The presence of erythrocytes in atherosclerotic lesions from apoE-deficient mice. It has been shown that apoE-deficient mice spontaneously develop atherosclerotic lesions of different stages with features similar to humans (12, 13). When the aortic samples from a mouse at age of 6 month old were examined by scanning electron microscopy, it was clearly shown that the integrity of endothelium was devastated by the formation of atherosclerotic lesions (Fig. 1a). The disruption of the endothelial lining was clearly seen around the plaques under the higher magnification (Fig. 1b). Furthermore, the disc-shape erythrocytes were found to reside in the hollow. Occasionally, the erythrocytes appeared in close proximity to the macrophages within the lesions (Fig. 1c). When the lesions were further examined by transmission electron microscopy, the translocation of erythrocytes, which could be easily recognized by their dark and dense bodies, in subendothelial space was clearly seen (Fig. 2a). These erythrocytes were deformed and some were readily engulfed by the foam cell-like macrophage (Fig. 2b).

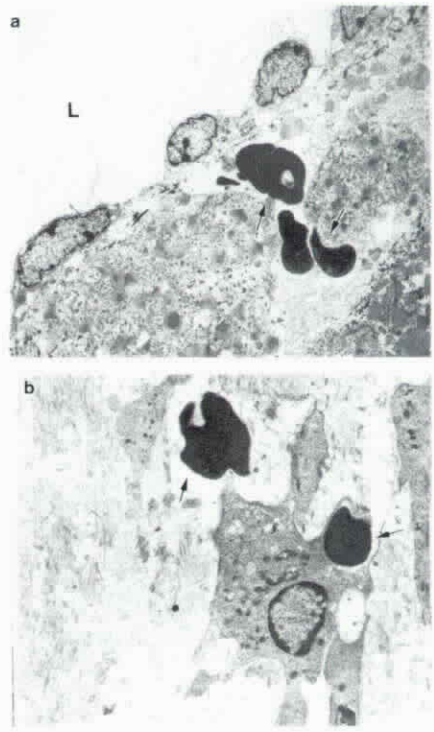

Fig. 2. Transmission electron microscopy of the atherosclerotic lesions from a 7 month old apoE-deficient mouse. L, lumen. Erythrocytes are pointed by arrows. Magnification, X2,500 (a), X6,000 (b).

Colocalization of erythrocytes and iron deposits in atherosclerotic lesions from cholesterol-fed rabbits. When the lesions from the heterozygous WHHL rabbits fed with high-cholesterol diet were examined, similar results were obtained. As shown in Fig. 3, the

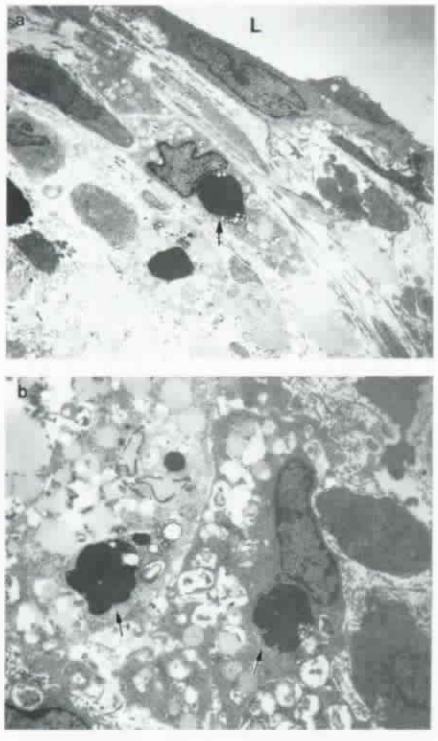

Fig. 3. Transmission electron microscopy of the atherosclerotic lesions from a heterozygous WHHL rabbit fed with high-cholesterol diet for 2 months. L, lumen. Erythrocytes are pointed by arrows. Magnification, X2,000 (a), X2,500 (b).

recently phagocytosed erythrocytes were evident in the cytoplasm of foam cells in lesions. When the paraffin-embedded sections were stained by hematoxylin-eosin Y, it was clearly shown that the erythrocytes, which were easily identified by their pink stains, were present in the plaque without sign of neovascularization or hemorrhage (Fig. 4a). Perls' staining conducted on a serial section demonstrated that iron deposit appeared in the same region rich in erythrocytes (Fig. 4b), suggesting that the iron released from digested hemoglobin may contribute to the iron deposited in lesions.

Colocalization of iron deposit and immunoreactivities of hemoglobin, HO-1 and ferritin in lesions from humans and apoE-deficient mice. When the sections of a human atherosclerotic lesion of intermediate stage were immunostained with specific antibody to hemoglobin, it was interesting to notice that the immunoreactivity was evident in macrophage-derived foam cells in lesions (Figs. 5d and 5h), indicating the degradation of erythrocytes within these cells. When the serial sections from the same lesion were immunostained with antibodies to HO-1 and ferritin, it was very interesting to find that a large portion of the immunoreactivity to HO-1 was localized in macrophages which were also positive with immuno-reactivities to hemoglobin and ferritin (Figs. 5e and 5f). When Perls' reaction was performed on the serial section from the same lesion, the positive brown iron stain was visible in the region with positive immunostain for hemoglobin (Fig. 5b). The colocalization of iron deposit and immunoreactivities of hemoglobin, HO-1 and ferritin was also observed in macrophages of atherosclerotic lesions from apoE-deficient mice (Fig. 6). Together, these results strongly support that hemoglobin released from the disrupted erythrocytes is at least one of the sources of iron present in vascular walls in the pathophysiological states.

Discussion

Oxidative stress is believed to play an important role in the pathogenesis of vascular diseases (1, 14). The oxidation of LDL in subendothelial intima leads to the formation of a variety of products with diverse biological activities, which exert profound effects on the development of the plaques. On the other hand, the free oxygen radicals generated from the oxidative events inevitably causes severe tissue injury. Apparently, it is of importance to unravel the underlying mechanism(s) how the oxidative reaction

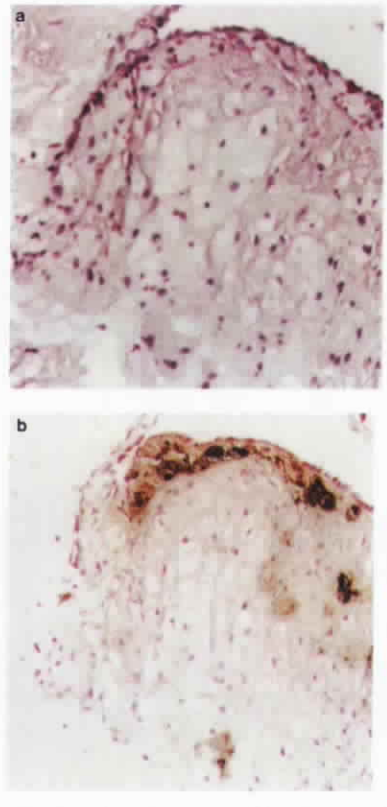

Fig. 4. Colocalization of erythrocytes and iron deposits in atherosclerotic lesions from rabbit. Consecutive paraffin sections of a lesion from heterozygous WHHL rabbit fed with high-cholesterol diet were subjected to (a) staining with hematoxylin and eosin and (b) Perls' reaction, respectively. Magnification, X 200 (a and b).

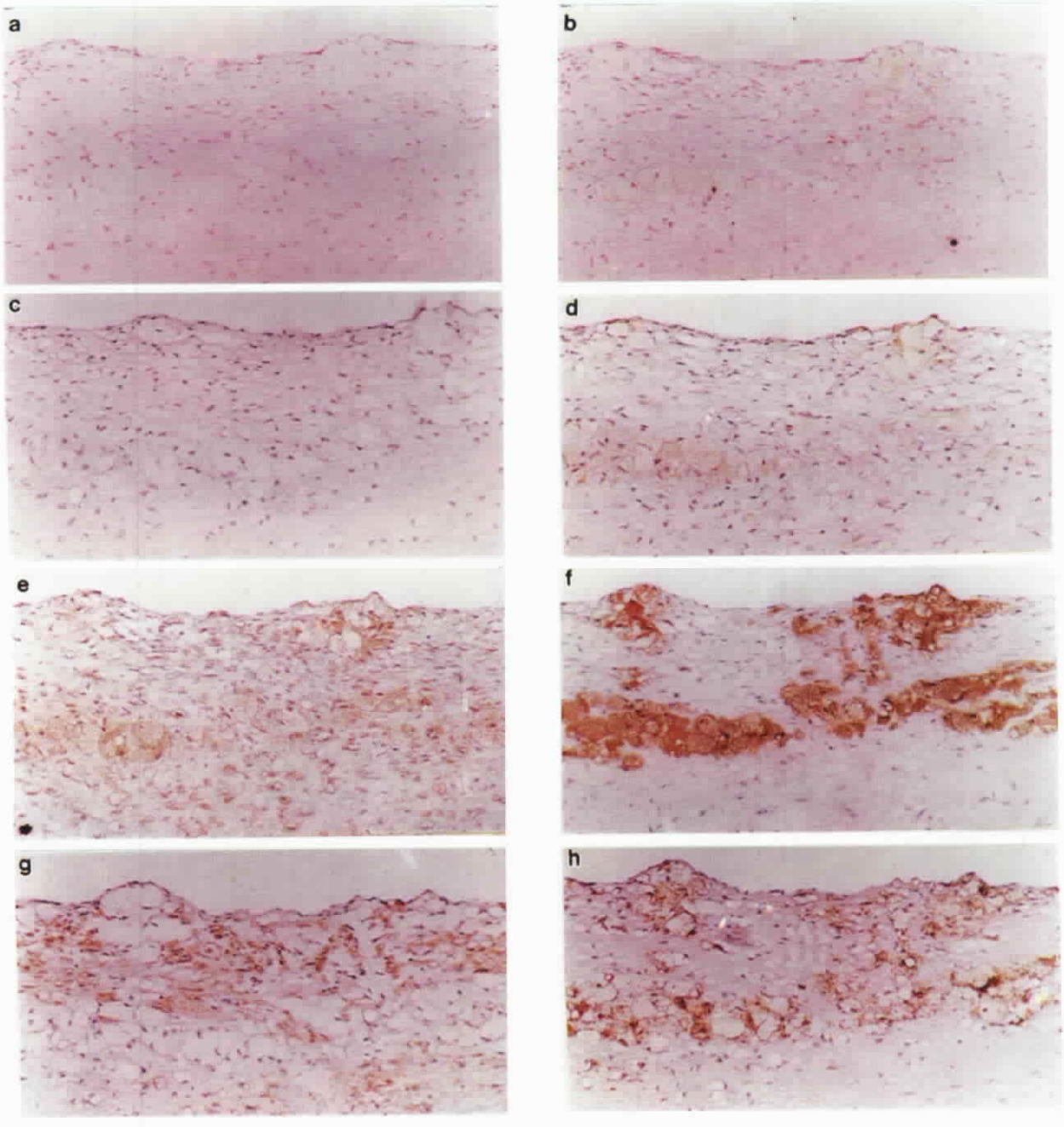

Fig. 5. Colocalization of iron and immunoreactivities of hemoglobin, HO-1 and ferritin in lesion from human patient. The serial sections of a lesion from a human patient (male, 16 year old) were subjected to (b) Perls' iron staining or immunostaining with antibodies to (d) hemoglobin (dilution 1:200), (e) HO-1 (dilution 1:200), (f) ferritin (dilution 1:200), (g) smooth muscle cell α-actin (dilution 1:50) and (h) macrophage CD68 antigen (dilution 1:100). The negative controls for Perls' reaction and immunostaining were shown in (a) and (c), respectively. Magnification, X100 (a-h).

is initiated and propagated in arterial walls in vivo. Recently, our laboratory and others reported that substantial amount of iron is present in human atherosclerotic lesions (8-11). Since iron is the most important catalyst for the generation of hydroxyl free radicals and the propagation of lipid oxidation in vivo, this observation suggests that iron may play a role in the oxidative reactions in plaques. Although the direct evidence is lacking, our recent study showing that ceroids, which are insoluble complexes of proteins/oxidized lipids commonly observed in lesions, are colocalized with iron deposits in human atherosclerotic lesions provided the first clue to support this notion (11). Nevertheless, the origin of iron deposited in lesions remained as a puzzle. In present study, we were able to provide the histological evidence to demonstrate that erythrocytes are present in atherosclerotic lesions. As clearly shown by the

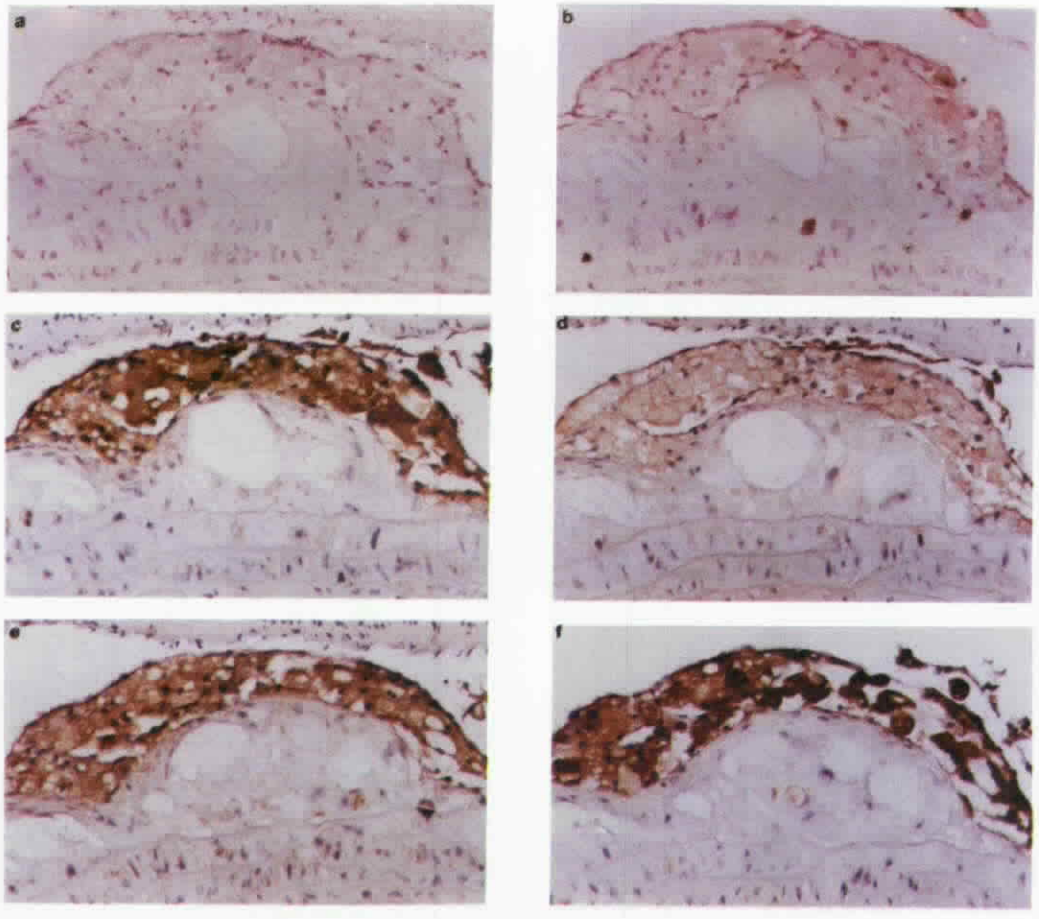

Fig. 6. Colocalization of iron and immunoreactivities of hemoglobin, HO-1 and ferritin in lesion from apoE-deficient mouse. The serial sections of a lesion from a 5 month old apoE-deficient mouse were subjected to (b) Perls' iron staining, or immunostaining with antibodies to (c) macrophage F4/80 antigen (dilution 1:200), (d) hemoglobin (dilution 1:200), (e) HO-1 (dilution 1:200), and (f) ferritin (dilution 1:200). The negative control for Perls' reaction was shown in (a). Section incubated with secondary antibody alone was negative (data not shown). Magnification, X200 (a-f).

transmission electron microscopy, the circulating erythrocytes, which can be easily identified by their distinctive features, are present in the subendothelial intima of atherosclerotic lesions of the apoE-deficient mice. Since most of them appeared to be readily phagocytosed by adjacent macrophages, that may explain the low appearance of these cells in lesions. The erythrophagocytosis was also evident in lesions from rabbits, indicating it is a universal event occurring in the course of lesion development. The degradation of phagocytosed erythrocytes was further demonstrated by the positive immunoreactivity to hemoglobin in macrophages of atherosclerotic lesions from humans and apoE-deficent mice. It is conceivable that the hemoglobin/heme released from the degraded erythrocytes would significantly increase the oxidative stress of the local tissues and exacerbate the progression of the plaques. Very recently, we reported that the stress-inducible protein, HO-1, is highly expressed in macrophages and smooth muscle cells of atherosclerotic lesion (15). Further experiments demonstrated that oxidized LDL is a potent activator for HO-1 gene induction in vitro(15). Since heme/hemoglobin are potent inducer for HO-1 gene expression (16, 17), it is very likely that part of the HO-1 induction, particularly in macrophages, may be caused by the heme stress. As revealed by immunohistochemical experiments carried out on serial sections, part of the HO-1 immunostains were detected in macrophages that also exhibited positive immunoreactivity to hemoglobin. The coinduction of HO-1 and the iron sequestering protein, ferritin, by oxidative stress was previously demonstrated in studies of cultured cells (18, 19). Since ferritin has been shown to be highly expressed and its distribution to be closely associated with the iron deposition in human atherosclerotic lesions (10), it is intriguing to reveal the correlation between the HO-1 and ferritin gene expression in atherosclerotic lesions. When the serial sections of same lesions from humans or apoE-deficient mice were subjected to Perls'reaction and immunostaining with antibody to ferritin, it was very

interesting to find that the positive iron stains were colocalized with immunoreactivities to hemoglobin, HO-1 and ferritin. Results obtained from the present study strongly implicate that the heme iron derived from the disrupted erythrocytes contributes to, at least in part, the iron accumulated in atherosclerotic lesions.

Although the histological presence of erythrocytes in lesions is evident, it is not clear how the translocation occurs. As revealed by the scanning electron microscopy, the integrity of endothelium of atherosclerotic vessels was damaged. It is very likely that the circulating erythrocytes are passively trapped into the arterial walls via the leaky junctions or gaps around the atherosclerotic plaques. Although normal erythrocytes do not adhere to endothelium, erythrocytes from patients with diabetes mellitus, sickle cell anemia and malaria have been shown to exhibit increased adhesion to endothelium and contributes to the pathogenesis of vascular complications (20-23). In the present case, it is not clear whether the adherence to endothelium is prerequisite to the transmigration of erythrocytes into subendothelial intima. It is intriguing to examine whether erythrocytes from patients or experimental animals with atherosclerosis are sticky to endothelium. In summary, the present study clearly demonstrated that circulating erythrocytes are present in atherosclerotic lesions. The erythrophagocytosis by macrophages followed by the release of hemoglobin/heme apparently leads to the increase in the abundance of iron in arterial walls, which would then aggravate the progression of the lesions by accelerating the iron-mediated oxidative reactions.

Acknowledgments

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