

ORIGINAL ARTICLE

Intraplaque Hemorrhage and Progression of Coronary Atheroma

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ABSTRACT

BACKGROUND

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Intraplaque hemorrhage is common in advanced coronary atherosclerotic lesions. The relation between hemorrhage and the vulnerability of plaque to disruption may involve the accumulation of free cholesterol from erythrocyte membranes.

METHODS

We stained multiple coronary lesions from 24 randomly selected patients who had died suddenly of coronary causes with an antibody against glycophorin A (a protein specific to erythrocytes that facilitates anion exchange) and Mallory's stain for iron (hemosiderin), markers of previous intraplaque hemorrhage. Coronary lesions were classified as lesions with pathologic intimal thickening, fibrous-cap atheromas with cores in an early or late stage of necrosis, or thin-cap fibrous atheromas (vulnerable plaques). The arterial response to plaque hemorrhage was further defined in a rabbit model of atherosclerosis.

RESULTS

Only traces of glycophorin A and iron were found in lesions with pathologic intimal thickening or fibrous-cap atheromas with cores in an early stage of necrosis. In contrast, fibroatheromas with cores in a late stage of necrosis or thin caps had a marked increase in glycophorin A in regions of cholesterol clefts surrounded by iron deposits. Larger amounts of both glycophorin A and iron were associated with larger necrotic cores and greater macrophage infiltration. Rabbit lesions with induced intramural hemorrhage consistently showed cholesterol crystals with erythrocyte fragments, foam cells, and iron deposits. In contrast, control lesions from the same animals had a marked reduction in macrophages and lipid content.

CONCLUSIONS

By contributing to the deposition of free cholesterol, macrophage infiltration, and enlargement of the necrotic core, the accumulation of erythrocyte membranes within an atherosclerotic plaque may represent a potent atherogenic stimulus. These factors may increase the risk of plaque destabilization.

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A THEROSCLEROTIC PLAQUE COMPRISES a heterogeneous mixture of cellular and acellular elements. The conversion of a stable, asymptomatic lesion to an unstable, ruptured plaque involves many processes, the most studied of which are inflammation, cellular breakdown, and expansion of the acellular, lipid-rich, necrotic core. It is commonly held that the death of macrophages and smooth-muscle foam cells, in addition to the aggregation of lipoproteins, contributes to the accumulation of extracellular free cholesterol within unstable plaques.^{1,2} The contribution of intraplaque hemorrhage to the expansion of the core of plaques, however, has not been studied, despite the knowledge that intraplaque hemorrhage is a common phenomenon. Furthermore, the free cholesterol content of erythrocyte membranes exceeds that of all other cells in the body, with lipid constituting 40 percent of the weight.^{3,4} Extravasated erythrocytes outside the coronary vasculature contain free cholesterol and macrophages,^{5,6} and intimal plaques in patients with pulmonary hypertension contain erythrocyte membranes.⁷

The aim of this study was to demonstrate erythrocyte membranes within the necrotic cores of human atherosclerotic plaques, even those without recent hemorrhages, and relate them to the progression and instability of the lesions. We also examined the fate of erythrocytes in established plaques in atherosclerotic rabbits to provide a model of hemorrhage-induced progression of lesions. Establishment of a link between intraplaque hemorrhage and the expansion of the lesions would provide another potential mechanism of plaque progression and vulnerability.

METHODS

SELECTION OF PATIENTS

An institutional review board approved the study. Hearts of patients who had died suddenly of coronary causes were obtained as described previously.⁸ Of 270 such patients, 100 were randomly selected for investigation to determine the incidence of hemorrhage in nonculprit plaques with luminal narrowing of more than 50 percent. The mean (\pm SD) number of intraplaque hemorrhages per heart was 5.0 ± 0.4 in patients with coronary thrombosis resulting from acute plaque rupture, as compared with 0.6 ± 0.3 in those with thrombosis caused by plaque erosion ($P < 0.002$) and 2.8 ± 0.8 in those with steno-

sis of at least 75 percent of the lesion in the absence of acute thrombi ($P < 0.04$).

The high incidence of intraplaque hemorrhage in hearts with ruptured plaques prompted further study of lesions in another 24 randomly selected patients who had died suddenly of coronary causes. In each case, if the necrotic core showed a predominance of erythrocytes on conventional staining with hematoxylin and eosin, the sections were excluded from analysis. The remaining sections were examined for glycophorin A, a protein specific to erythrocytes that facilitates anion exchange.⁹ Nonculprit plaques were categorized morphologically to gain insights into the role of intraplaque hemorrhage in the progression and instability of lesions. Finally, we reviewed patients' records from our institute to identify those with nonvascular lesions containing hemorrhagic areas associated with pericarditis, hemangiomas, and cholesterol granulomas of the lung.

TISSUE PROCESSING

Formalin-fixed coronary segments were embedded in paraffin, and 4- μ m sections were stained with hematoxylin and eosin and Movat pentachrome. Parallel sections were prepared to identify collagen matrix (with the use of picrosirius red stain) and iron (with the use of Mallory's stain), as well as for immunohistochemical analysis.

CLASSIFICATION OF LESIONS

We examined 810 sections for lesions and classified the lesions using an American Heart Association scheme modified by our laboratory.¹⁰ We included lesions with pathologic intimal thickening, fibroatheromas whose cores were in an early stage ("early core") or late stage ("late core") of necrosis, and thin-cap fibroatheromas. Early cores contain cholesterol clefts, macrophages, and proteoglycans or collagen; late cores have more numerous cholesterol clefts and cellular debris with an absence of extracellular matrix. Using these criteria, we analyzed 365 plaques: 129 plaques with pathologic intimal thickening, 79 fibroatheromas with early cores, 105 fibroatheromas with late cores, and 52 thin-cap fibroatheromas.

IMMUNOHISTOCHEMICAL STUDIES

Paraffin sections were incubated with an antibody against glycophorin A to identify sites of previous plaque hemorrhage. Macrophages were identified by staining with antibody against CD68, and endo-

thelial cells by staining with antibody against von Willebrand factor. Primary antibodies were labeled with a biotinylated link antibody directed against mouse antigen with the use of a peroxidase-based kit (LSAB, Dako) and visualized with use of a 3-amino-9-ethylcarbazole substrate.

The percentage of the necrotic core or lipid pool that was made up of glycophorin A and iron was graded semiquantitatively by two independent observers using a scale from 0 to 4, with higher scores indicating higher percentages. Only specimens with staining of erythrocyte fragments were included in the analysis. Computer-based morphometry was used to measure the size of the lipid core, plaque area, and macrophage content as described previously.¹¹

PROOF-OF-CONCEPT STUDY IN RABBITS

Rabbit Model of Simulated Intraplaque Hemorrhage

Six New Zealand white rabbits that were three to four months old were fed an atherogenic diet (containing 1 percent cholesterol and 6 percent peanut oil) to initiate the formation of atheromas. After one week, lesions were produced in the abdominal aorta by balloon-induced injury. The animals were fed the atherogenic diet for another 4 weeks and then given purified rabbit chow with no added cholesterol until they were killed at 14 weeks.

Rabbit Model of Intramural Hemorrhage

After eight weeks of the nonatherogenic diet, the rabbits underwent a left-sided lateral laparotomy while under general anesthesia, and a 4-cm segment of the abdominal aorta was exposed. A 30-gauge

needle was introduced into the arterial lumen at the sites of lesions and then slowly withdrawn until the beveled tip was within the arterial wall. Washed autologous erythrocytes (25 to 50 μ l) were slowly delivered into established atherosclerotic plaques with the use of a handheld 1-ml syringe. A slightly raised hematoma provided visual confirmation that erythrocytes were trapped within the plaque. Injections were made in two to three lesions per animal; non-injected lesions served as controls. The animals continued to be fed a normal-chow diet for another six weeks.

Tissue Preparation and Staining

The rabbits were killed, the arterial tree was perfused and fixed, and the abdominal aorta was excised and cut into 3-mm segments. Frozen blocks were prepared, and cryosections (6 μ m) were stained with hematoxylin and eosin and Movat pentachrome. Additional sections were used to determine the lipid content (with oil red O stain) and the iron content and for immunohistochemical analysis. Macrophages were identified with a specific monoclonal antibody against rabbit alveolar macrophages (RAM11). To identify erythrocyte membranes, we stained the sections with isolectin B4 from *Bandeiraea simplicifolia* conjugated with biotin.¹²

STATISTICAL ANALYSIS

Data are presented as means \pm SE. We used a t-test with Dunnett's correction to compare continuous variables by analysis of variance. Differences between measured variables were considered significant if the resultant P value was 0.05 or less.

Table 1. Morphometric Analysis of 365 Plaques in Coronary Arteries from Patients Who Died Suddenly of Coronary Causes.*

Type of Plaque	No. of Plaques	Glycophorin A Score†	Iron Score‡	Size of Necrotic Core	Extent of Macrophage Infiltration
					<i>mm</i> ²
Plaque with pathologic intimal thickening but with no necrotic core	129	0.09 \pm 0.04	0.07 \pm 0.05	—	0.002 \pm 0.001
Fibroatheroma					
Core in early stage of necrosis	79	0.23 \pm 0.07	0.17 \pm 0.08	0.06 \pm 0.02	0.018 \pm 0.004
Core in late stage of necrosis	105	0.94 \pm 0.11‡	0.41 \pm 0.09‡	0.84 \pm 0.08‡	0.059 \pm 0.007‡
Thin-cap fibroatheroma	52	1.60 \pm 0.20‡	1.24 \pm 0.24‡	1.95 \pm 0.30‡	0.142 \pm 0.016‡

* Plus–minus values are means \pm SE.

† Scores can range from 0 to 4, with higher scores indicating greater proportions of the analyte.

‡ P<0.001 for the comparison with fibroatheromas whose cores were in an early stage of necrosis.

RESULTS

HUMAN CORONARY PLAQUES

Hemorrhage in Coronary Plaques

Table 1 shows the glycoprotein A and iron scores, the sizes of the necrotic cores, and the extent of macrophage infiltration in the specific types of lesions. As compared with lesions with pathologic intimal thickening or early-core fibroatheromas, fibroatheromas with late cores and thin-cap fibroatheromas had significantly greater mean scores for glycoprotein A and iron ($P < 0.001$). Previous hemorrhage was identified in 8 of 129 plaques with pathologic intimal thickening (6 percent), 15 of 79 fibroatheromas with early cores (19 percent), and 56 of 105 fibroatheromas with late cores (53 percent). The incidence of hemorrhage was greatest among thin-cap fibroatheromas, with 40 of 52 lesions (77 percent) positive for glycoprotein A or iron. Advanced lesions with erythrocytes often contained extensive areas of

neovascularization, with diffuse perivascular staining for von Willebrand factor (Fig. 1). The area of the necrotic core was significantly greater in fibroatheromas with late cores or thin caps than in fibroatheromas with early cores ($P < 0.001$). The larger necrotic cores were associated with an increased density of CD68-positive macrophages, especially toward the fibrous cap. Notably, higher glycoprotein A or iron scores were associated with larger necrotic cores (Fig. 2).

Hemorrhage in Noncoronary Lesions

Selected specimens from patients with hemorrhage in noncoronary lesions were examined. In one example, a right atrial hemangioma contained a large collection of erythrocytes, cholesterol clefts, and foamy cells; perivascular staining for von Willebrand factor was also evident. In a specimen from a patient with hemorrhagic pericarditis, there was intense staining for glycoprotein A with extensive de-

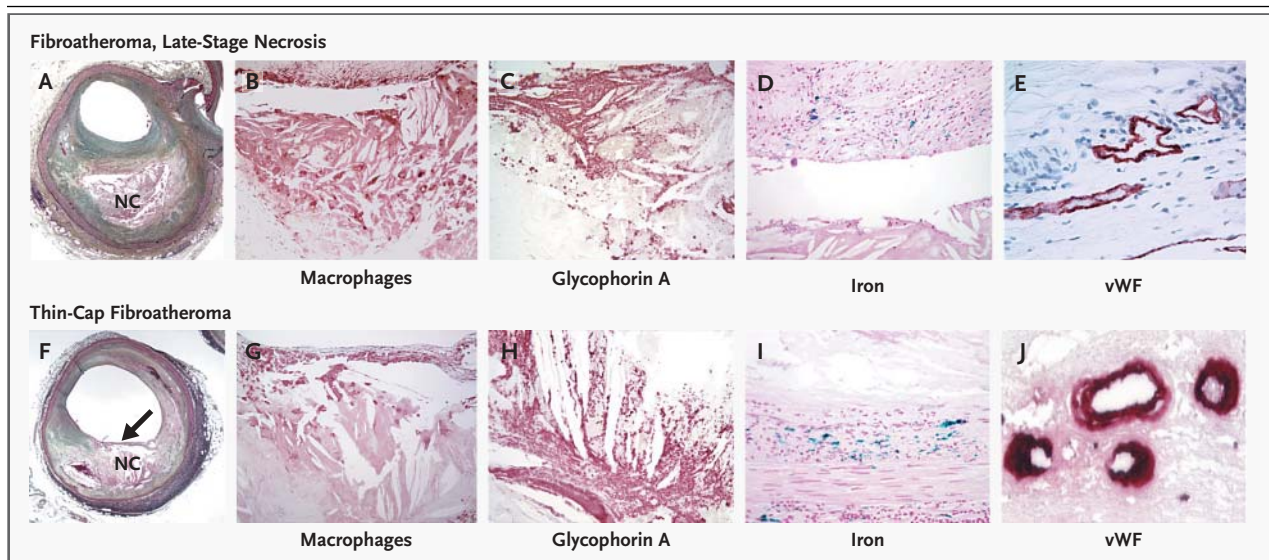
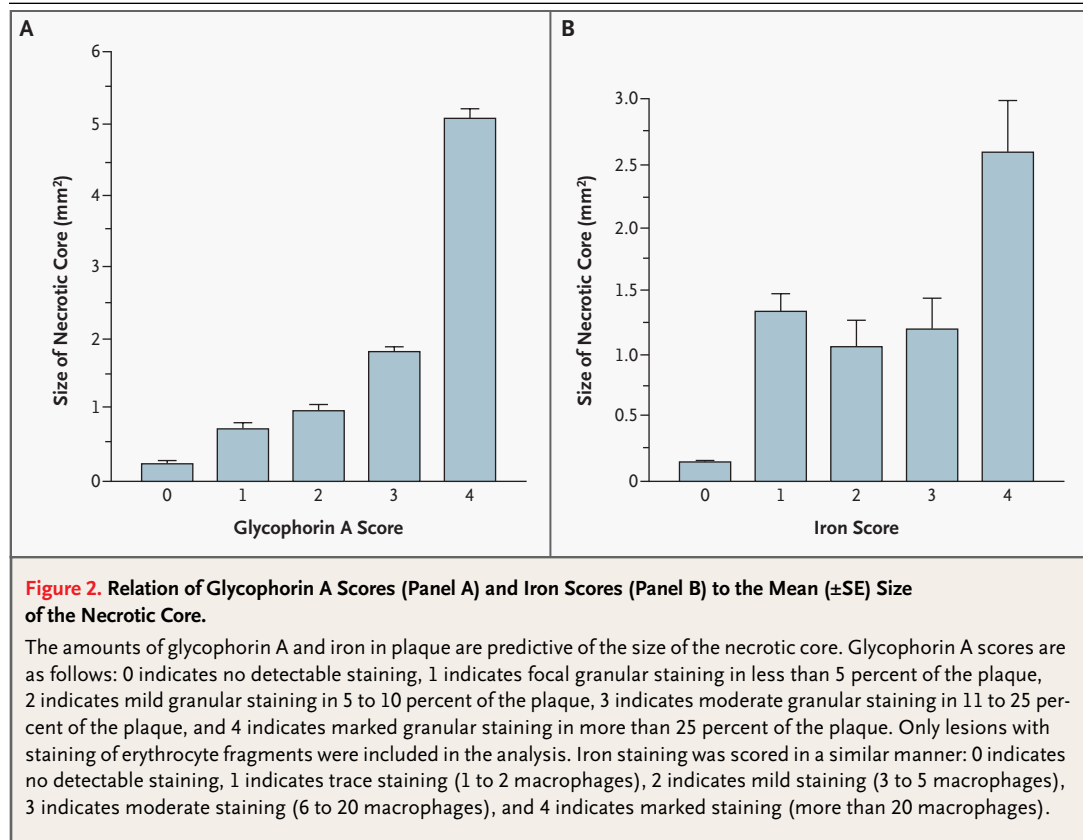


Figure 1. Intraplaque Hemorrhage in Fibroatheroma with a Core in a Late Stage of Necrosis (Panels A, B, C, D, and E) and Thin-Cap Fibroatheroma (Panels F, G, H, I, and J).

Panel A shows a low-power view of a fibroatheroma with a late-stage necrotic core (NC) (Movat pentachrome, $\times 20$). Panel B shows intense staining of CD68-positive macrophages within the necrotic core ($\times 200$). Panel C shows extensive staining for glycoprotein A in erythrocyte membranes localized with numerous cholesterol clefts within the necrotic core ($\times 200$). Panel D shows iron deposits (blue pigment) within foam cells (Mallory's stain, $\times 200$). Panel E shows microvessels bordering the necrotic core with perivascular deposition of von Willebrand factor (vWF) ($\times 400$). Panel F shows a low-power view of a fibroatheroma with a thin fibrous cap (arrow) overlying a relatively large necrotic core (Movat pentachrome, $\times 20$). The fibrous cap is devoid of smooth-muscle cells (not shown) and is heavily infiltrated by CD68-positive macrophages (Panel G, $\times 200$). Panel H shows intense staining for glycoprotein A in erythrocyte membranes within the necrotic core, together with cholesterol clefts ($\times 100$). Panel I shows an adjacent coronary segment with iron deposits (blue pigment) in a macrophage-rich region deep within the plaque (Mallory's stain, $\times 200$). Panel J shows diffuse, perivascular deposits of von Willebrand factor in microvessels, indicating that leaky vessels border the necrotic core ($\times 400$).



position of free cholesterol in the absence of inflammatory cells; however, CD68-positive macrophages and extracellular iron deposits were present in the periphery of the lesion (Fig. 3).

RABBIT MODEL OF INDUCED INTRAMURAL HEMORRHAGE

Plasma Lipids

The mean total cholesterol levels in the rabbits were 45 ± 19 mg per deciliter (1.2 ± 0.5 mmol per liter) before the experiment was initiated and 2216 ± 466 mg per deciliter (57.3 ± 12.0 mmol per liter) after five weeks of the atherogenic diet. At the time of the erythrocyte injection (after eight weeks of a control diet devoid of supplemental lipids), the cholesterol levels were dramatically lower and thereafter were equivalent to base-line levels (33 ± 10 mg per deciliter [0.9 ± 0.3 mmol per liter]).

Histologic Appearance of Aortic Lesions with Intramural Hemorrhage

Rabbit atheromas with injected erythrocytes had more extensive macrophage infiltration than control lesions (17 ± 3.6 percent vs. 3.7 ± 2.2 percent,

$P=0.03$), despite the fact that the sizes of the plaques were similar in the two groups. The lipid content on staining with oil red O was also significantly higher in the plaques with injected erythrocytes than in control lesions (34 ± 6 percent vs. 22.1 ± 4.5 percent, $P=0.05$). Histologically, plaques with injected erythrocytes showed discrete dissection planes with circumferential infiltration of macrophages within the arterial wall.

Numerous lipid-laden RAM11-positive foam cells were found in the superficial and deep layers of the arterial wall. The lipid content was extensive in plaques that had received an injection of erythrocytes. Cholesterol crystals were frequently found with erythrocyte fragments (as evidenced by staining with isolectin B4) and macrophage foam cells containing iron deposits (Fig. 4). In contrast, control lesions contained smooth-muscle cells, particularly toward the areas of the lumen and media and collagen matrix. Furthermore, control lesions had fewer macrophages in parallel with reduced staining for oil red O, and the erythrocytes were confined to the adventitial layer (Fig. 4).

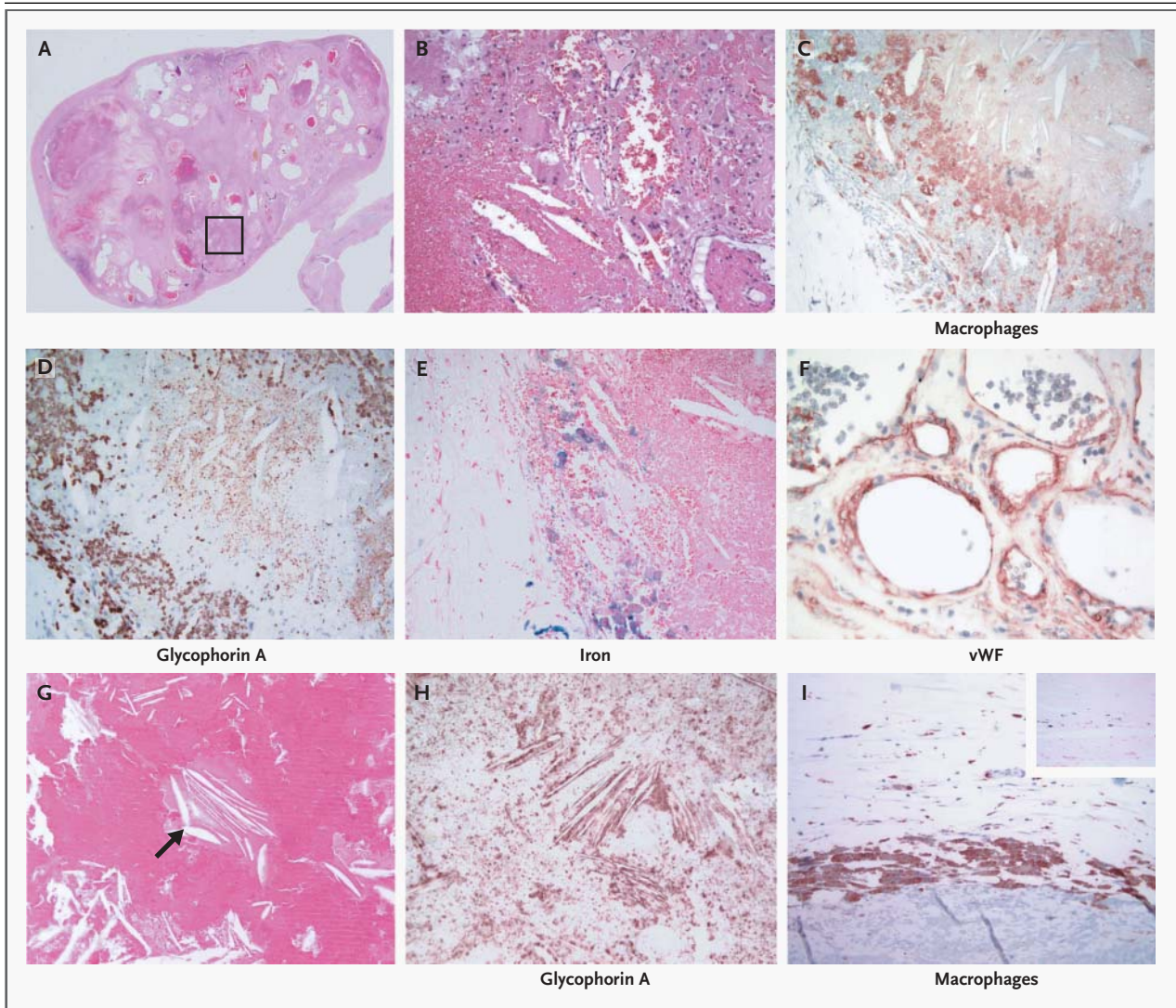


Figure 3. Atherogenic Changes Associated with Extravasated Erythrocytes in Noncoronary Lesions.

Panel A shows a section of a right atrial hemangioma stained with hematoxylin and eosin ($\times 4$). Panel B shows a higher-power magnification of the area in the black box in Panel A, in which a large number of erythrocytes as well as cholesterol clefts are present ($\times 200$). Panel C shows intense staining for CD68-positive macrophages in an area of extravasated erythrocytes ($\times 200$). Panel D shows intact erythrocytes and membrane remnants identified on the basis of staining with antibody against glycophorin A ($\times 200$). Panel E shows iron deposits (blue pigment), macrophages, and cholesterol clefts (Mallory's stain, $\times 200$). Panel F shows perivascular accumulation of von Willebrand factor (vWF) in capillaries ($\times 400$). Panel G shows a section of pericardium (from a patient with acute hemorrhagic pericarditis) containing erythrocytes and cholesterol clefts (arrow, $\times 100$). Panel H shows the results of staining with antibody against glycophorin A in a region similar to that shown in Panel G ($\times 400$). Erythrocyte membranes and crystalline cholesterol are present, but macrophages are absent. Panel I shows the presence of macrophages at the periphery of accumulated erythrocytes; staining for iron (Mallory's stain; inset, $\times 400$) was also noted.

DISCUSSION

Our findings indicate that there is an association among intraplaque hemorrhage, an increase in the size of the necrotic core, and lesion instability in coronary plaques. Immunostaining with antibody

against glycophorin A revealed previous hemorrhages in lesions with late cores and those prone to rupture. The degree of reactivity of glycophorin A and the level of iron accumulation corresponded to the size of the necrotic core, and the increase in these variables paralleled the increase in the densi-

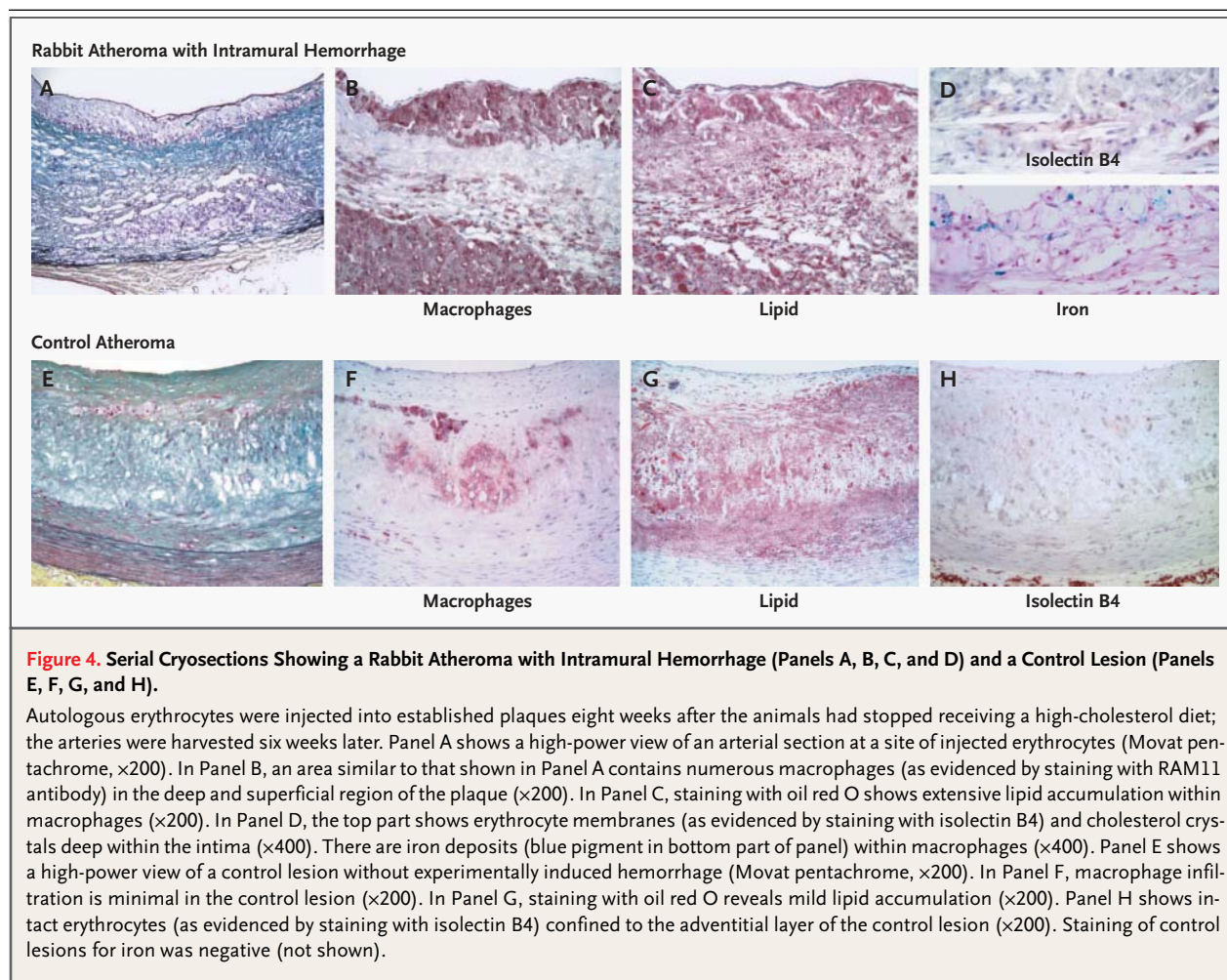


Figure 4. Serial Cryosections Showing a Rabbit Atheroma with Intramural Hemorrhage (Panels A, B, C, and D) and a Control Lesion (Panels E, F, G, and H).

Autologous erythrocytes were injected into established plaques eight weeks after the animals had stopped receiving a high-cholesterol diet; the arteries were harvested six weeks later. Panel A shows a high-power view of an arterial section at a site of injected erythrocytes (Movat pentachrome, $\times 200$). In Panel B, an area similar to that shown in Panel A contains numerous macrophages (as evidenced by staining with RAM11 antibody) in the deep and superficial region of the plaque ($\times 200$). In Panel C, staining with oil red O shows extensive lipid accumulation within macrophages ($\times 200$). In Panel D, the top part shows erythrocyte membranes (as evidenced by staining with isolectin B4) and cholesterol crystals deep within the intima ($\times 400$). There are iron deposits (blue pigment in bottom part of panel) within macrophages ($\times 400$). Panel E shows a high-power view of a control lesion without experimentally induced hemorrhage (Movat pentachrome, $\times 200$). In Panel F, macrophage infiltration is minimal in the control lesion ($\times 200$). In Panel G, staining with oil red O reveals mild lipid accumulation ($\times 200$). Panel H shows intact erythrocytes (as evidenced by staining with isolectin B4) confined to the adventitial layer of the control lesion ($\times 200$). Staining of control lesions for iron was negative (not shown).

ty of macrophages, raising the possibility that the hemorrhage itself serves as an inflammatory stimulus. To test the hypothesis that erythrocytes actively participate in the progression of atheromas, we devised an experimental model of intramural hemorrhage in the rabbit. The injection of autologous erythrocytes into existing lesions produced plaques with crystalline cholesterol, lipid, iron, and foam cells, whereas control lesions had little free cholesterol and few macrophages. The finding that intramural hemorrhage in an experimental atherosclerotic lesion induces the formation of cholesterol crystals with the recruitment of macrophages supports our hypothesis that erythrocyte membranes in the necrotic core of human coronary lesions can cause an abrupt increase in the levels of free cholesterol, resulting in expansion of the necrotic core and the potential for the destabilization of plaque.

In the first half of the 20th century, Wartman and others suggested that intraplaque hemorrhage is a major contributor to the progression of coronary lesions.¹³⁻¹⁵ Studies involving the injection of silicon polymer into atherosclerotic human coronary arteries¹⁶ demonstrated an elaborate microvascular network (the vasa vasorum) extending from the adventitia through the media and into the thickened intima; nonatherosclerotic vessels rarely had vasa vasorum. Intraplaque hemorrhage is believed to arise from the disruption of thin-walled microvessels that are lined by a discontinuous endothelium without supporting smooth-muscle cells.¹⁷ Moreover, several investigators, including some from our laboratory, have suggested that intraplaque hemorrhage and rupture of the fibrous cap are associated with an increased density of microvessels.¹⁸⁻²⁰ A greater number of vasa vasorum in ruptured plaques

and hemorrhages suggests that a larger pool of erythrocytes is available to participate in necrotic-core enlargement within these lesions. Our finding of diffuse, perivascular staining of vasa vasorum with von Willebrand factor and evidence of erythrocyte membranes within necrotic cores points to microvascular disruption as a source of erythrocyte-derived cholesterol. Plaque fissures could also account for the accumulation of erythrocytes; however, fissures are often accompanied by luminal thrombi, which we did not find in any specimen.

Lipid composition is believed to influence the stability of atherosclerotic plaques, given that the level of free cholesterol is significantly increased in disrupted lesions.²¹ Furthermore, the percentage of cholesterol clefts is greater in lesions that have ruptured than in fibrocalcific plaques.¹⁰ Although apoptotic macrophages may be a source of free cholesterol, it is unlikely that the total free cholesterol content in plaques could be derived from foam cells alone, since most of the cholesterol in foam cells is esterified.²² Our finding of both cholesterol crystals and glycophorin A in the necrotic cores of advanced coronary plaques is similar to the finding of cholesterol clefts, macrophages, and iron in large areas of extravasated erythrocytes outside the coronary circulation. Although it is conceivable that the cholesterol crystals in these nonvascular lesions are derived from foam cells, cholesterol clefts often reside exclusively in areas of erythrocytes lacking macrophages. In these instances, cholesterol derived from erythrocyte membranes may exceed a critical level, forming an immiscible cholesterol phase and ultimately crystallizing.²³

The cholesterol content of erythrocyte membranes may represent an independent risk factor for acute ischemic events, since it reflects the levels of circulating cholesterol.²⁴ In rats and rabbits, hypercholesterolemia causes the cholesterol content of erythrocyte membranes to increase substantially.^{25,26} The cholesterol content of erythrocyte membranes is also elevated in patients with familial hypercholesterolemia and decreases with short-term treatment with statins.^{27,28} The positive association between an elevated serum cholesterol level and an increased number of vulnerable plaques,⁸ together with our findings of intense staining for glycophorin A in advanced lesions and the dependence of erythrocyte-membrane cholesterol on circulating lipids, lends support to the hypothesis that the instability of plaques may be mediated in part by erythrocyte-membrane cholesterol.

The cellular response to extravasated erythrocytes appears to be influenced by the unique properties of the atherosclerotic intima. In the brain, skin, and normal arterial wall, experimental hematomas provoke a healing response characterized by the lysis of erythrocytes at 24 hours, followed within four to five days by an influx of macrophages, erythrophagocytosis, and the accumulation of iron.²⁹⁻³¹ Most of the hemorrhage in these tissues resolves by 7 to 14 days. In contrast, the intima of an atherosclerotic plaque appears to provide the appropriate milieu for the retention of erythrocyte-membrane cholesterol and foam cells.³² Hemorrhage within the necrotic core of a plaque may attract macrophages that eventually become trapped within the core and are unable to survive.

The signals erythrocytes may use to stimulate inflammation in coronary and noncoronary hemorrhages are not fully understood. The crystallization of cholesterol from erythrocyte membranes may incite a foreign-body reaction, as seen in cholesterol granulomas.³³ Alternatively, the migration of macrophages may be promoted by multispecific receptors on erythrocyte membranes, which can bind a wide array of chemokines in the blood, including monocyte chemoattractant peptide 1.^{34,35} Furthermore, the products of lipid oxidation from senescent erythrocytes or iron-catalyzed reactions may liberate potent chemoattractants.³⁶ On the basis of our experimental data in rabbits, acute hemorrhagic events promote the accumulation of free cholesterol and stimulate the excessive influx of macrophages. Our findings of intramural hemorrhage may further explain the episodic growth of human atherosclerotic plaque and may also explain why a coronary lesion can remain quiescent for extended periods and then suddenly become unstable.

Recent studies of carotid plaques suggest that lipids derived from erythrocyte membranes may contribute to the formation of foam cells. In an analysis of microvessels in carotid-endarterectomy specimens, Kockx et al. found excessive perivascular accumulation of von Willebrand factor, inducible nitric oxide synthase, and ceroid (a marker of previous oxidative events) within foam cells in 27 percent of plaques.³⁷ Macrophages frequently contain hemoglobin and iron, suggesting that phagocytosis of erythrocytes may contribute to the formation of foam cells.³⁸ Similar lipid-containing cells, expressing both ceroid and inducible nitric oxide synthase, have been generated in an atherosclerosis-free setting by incubating murine macrophages with oxi-

dized erythrocytes. Moreover, erythrophagocytosis as a result of microhemorrhages may have additional consequences; the iron accumulated from the breakdown of hemoglobin can act as a catalyst in the formation of free radicals, which may contribute to the modification of low-density lipoprotein cholesterol and cell death.^{39,40}

Our finding of erythrocyte membranes in the necrotic core of advanced coronary atheromas may widen our view regarding the origin of free chole-

sterol in developing vulnerable lesions. Erythrocyte membranes are capable of providing a substantial amount of lipid and may promote the recruitment of macrophages into the fibrous cap. Therefore, intraplaque hemorrhage represents a critical event in the induction of instability in these lesions.

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