

Remodeling of Saccular Cerebral Artery Aneurysm Wall Is Associated With Rupture

Histological Analysis of 24 Unruptured and 42 Ruptured Cases

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Background and Purpose—The cellular mechanisms of degeneration and repair preceding rupture of the saccular cerebral artery aneurysm wall need to be elucidated for rational design of growth factor or drug-releasing endovascular devices.

Methods—Patient records, preoperative vascular imaging studies, and the snap-frozen fundi resected after microsurgical clipping from 66 aneurysms were studied. Immunostainings for markers of smooth muscle cell (SMC) phenotype, proliferation, and inflammatory cell subtypes and TUNEL reaction were performed.

Results—Unruptured (24) and ruptured (42) aneurysms had similar dimensions (median diameter in unruptured 6 mm; median in ruptured 7 mm; $P=0.308$). We identified 4 basic types of aneurysm wall that associated with rupture: (1) endothelialized wall with linearly organized SMCs (17/66; 42% ruptured), (2) thickened wall with disorganized SMCs (20/66; 55% ruptured), (3) hypocellular wall with either myointimal hyperplasia or organizing luminal thrombosis (14/66; 64% ruptured), and (4) an extremely thin thrombosis-lined hypocellular wall (15/66; 100% ruptured). Apoptosis, de-endothelialization, luminal thrombosis, SMC proliferation, and T-cell and macrophage infiltration associated with rupture. Furthermore, macrophage infiltration associated with SMC proliferation, and both were increased in ruptured aneurysms resected <12 hours from rupture, suggesting that these were not just reactive changes.

Conclusions—Before rupture, the wall of saccular cerebral artery aneurysm undergoes morphological changes associated with remodeling of the aneurysm wall. Some of these changes, like SMC proliferation and macrophage infiltration, likely reflect ongoing repair attempts that could be enhanced with pharmacological therapy. (*Stroke*. 2004;35:2287-2293.)

Key Words: cerebral aneurysm ■ inflammation ■ intracranial aneurysm ■ rupture

Saccular cerebral artery aneurysms (SCAAs) in the cerebral artery bifurcations are the most common cause of subarachnoid hemorrhage (SAH).¹ Known SCAA risk factors include hypertension, smoking, heavy alcohol consumption, and female gender.^{2,3} Some SCAA cases are familial,⁴ with a linkage to 19q13.3 area in the Finnish population^{5,6} and to the 7q11 area in the Japanese population.⁷ The mechanisms of how these factors predispose to the formation or rupture of the SCAA wall are not known.

There are few reports about SCAA wall histology,⁸⁻¹³ and the cellular mechanisms of SCAA rupture are unknown. Characterization of these mechanisms is mandatory for development of targeted treatment to prevent SCAA growth and rupture. Such a treatment could be delivered either systemically or by using coated endovascular devices such as stents or coils.^{14,15}

SCAA wall is subjected to increasing hemodynamic stress and likely becomes unstable and undergoes morphological

changes before rupture. The cellular mechanisms of adaptation to increased hemodynamic stress in normal arterial wall are proliferation and luminal migration of smooth muscle cells (SMCs).^{16,17} These cellular mechanisms are partly controlled by cytokines released by inflammatory cells infiltrating the vascular wall.¹⁸ In addition to these 3 mechanisms, the SCAA wall is prone to form thrombosis lining of the luminal wall because of altered flow conditions.¹⁹ Organization of this thrombus lining thickens the wall.

Our aim was to characterize these cellular mechanisms in a series of human SCAA fundi (24 unruptured and 42 ruptured) resected after microsurgical clipping of the SCAA neck from patients that did not significantly differ in age or gender, or in aneurysm size or location.

Materials and Methods

Human SCAA Samples

SCAA samples were obtained during microsurgery by resecting the aneurysm sac distal to the clip closing the neck (J.H., M.N.,

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TABLE 1. Monoclonal Mouse Anti-Human Antibodies Used in the Study

Antigen	Clone	Dilution	Source
CD31 (PECAM 1 [platelet endothelial cell adhesion molecule 1]) ²⁰	JC70A	1:200	DAKO
Alfa-smooth muscle actin ²¹	1A4	1:500	Sigma
Myosin heavy chain ²²	SMMS-1	1:200	DAKO
Fibroblast marker ²³	5B5	1:200	DAKO
Ki67 ²⁴	MIB-1	1:250	DAKO
CD45 (LCA [leukocyte common antigen]) ²⁵	2B11+PD7/26	1:200	DAKO
CD3 (TCR [T-cell receptor])	PC3/188A	1:200	DAKO
CD11b (MAC 1 [macrophage adhesion molecule 1]) ²⁶	2LPM19c	1:200	DAKO
CD68 (macrophage marker) ²⁷	PG-M1	1:200	DAKO
CD163 (macrophage marker) ²⁸	Ber-MAC3	1:200	DAKO

Department of Neurosurgery, Helsinki University Central Hospital; years 2000 to 2002; 220 aneurysms operated per year). The tissue samples were snap-frozen in liquid nitrogen and stored in -70°C . The study was approved by the ethics committee of the Departments of Neurology, Neurosurgery, Otorhinolaryngology, and Ophthalmology at the Helsinki University Central Hospital.

Histology and Immunohistochemistry

Snap-frozen tissue samples were cryosectioned at $4\ \mu\text{m}$. For histology, sections were stained with hematoxylin-eosin (HE) and Weigert–van Gieson methods. For immunohistochemistry, sections were first incubated for 30 minutes at room temperature in PBS with 1.5% horse serum, and then overnight at 4°C with a monoclonal mouse anti-human primary antibody (Table 1) at 1:200 or 1:100 dilution in PBS with 1.5% horse serum. The primary antibody was detected after blocking endogenous peroxidase by 20 minutes incubation at room temperature in PBS with 0.1% hydrogen peroxide using the horseradish peroxidase–conjugated Vectastain anti-mouse kit (Vector Laboratories) and diaminobenzidine (Sigma-Aldrich). Sections were background stained with Gill's hematoxylin (Vector Laboratories). Substitution of the primary antibody with an irrelevant monoclonal antibody (anti-bromodeoxyuridine, clone BU20A; DAKO) or with PBS with 1.5% horse serum served as negative controls. Anonymized sections from human tonsils served as positive controls. TUNEL stainings were performed using the peroxidase-conjugated in situ cell death detection kit (Roche Diagnostics).

Histological Analysis

All histological quantifications were performed blinded for the rupture status. Two blinded observers (J.F., A. Paetau) performed classification of the SCAA wall structure. In addition, preservation of elastic laminae and endothelium, as well as the presence of atherosclerotic calcifications, medial layer degeneration, myointimal hyperplasia (MH), and endoluminal thrombosis, were evaluated (J.F.). To quantitate the number of fibroblasts and myofibroblasts, the number of fibroblast antigen+ cells was counted at $\times 40$ magnification from 2 active areas, and results are given as the mean value of the ratio of these cells and total cell number in the 2 active areas. To quantitate proliferation, the mean value of Ki67+ cells was quantitated similarly.

To quantitate inflammatory cell infiltration, the number of the CD45+, CD3+, CD68+, CD163+, and CD11b+ inflammatory cells was counted from a standardized grid area using $\times 40$ magnification ($0.0625\ \text{mm}^2$) from 2 active areas of the SCAA wall. The mean value for the density of positive cells (number of cells/grid area) was calculated. The ratios and densities were calculated separately for the SCAA wall and for the areas of MH/organizing thrombosis (OT) when present.

Clinical and Radiological Data

Clinical data were collected from the patients' medical records. Dimensions of the aneurysms were measured from preoperative vascular imaging studies: computed tomography, magnetic resonance angiography, or digital subtraction angiography.

Statistics

Statistics were calculated using the NCSS 2000 (NCSS Statistical Software). For categorical variables, proportions were calculated and χ^2 independence test was used. For numeric variables, median and range were calculated, and Mann–Whitney *U* test and Kruskal–Wallis multiple comparison test were used. Logistic regression and multiple linear regression were used in multivariable analysis. α -Level was 0.05.

Results

Patients and SCAAs

Patients with unruptured ($n=24$) or ruptured ($n=42$) SCAAs did not differ by age or gender (Table 2). The aneurysm neck and fundus sizes were similar (Table 2), and the most frequent locations were middle cerebral artery (67% in unruptured versus 41% in ruptured) and anterior communicating artery (ACoM; 8% in unruptured versus 21% in ruptured). Secondary pouches in preoperative angiographies were seen in 30% of unruptured and in 67% of ruptured SCAAs ($P=0.005$).

Histological Types of SCAA Walls

SCAA walls of the 6 familial aneurysm patients did not differ from the 60 sporadic ones. Lack of elastic laminae was a common feature in the SCAAs studied. Atherosclerotic calcifications were seen in only 5 unruptured and 2 ruptured cases. However, pads of MH or MH-like disorganized wall structure occurred in both groups (Table 2). Four basic types of SCAA wall structure were distinguished (Figure 1A through 1D): type A ($n=17$), endothelialized wall with linearly organized SMC; type B ($n=20$), thickened wall with disorganized SMC; type C ($n=14$), hypocellular wall with either MH or OT; and type D ($n=15$), an extremely thin thrombosis-lined hypocellular wall. The prevailing wall type in the sample significantly associated ($P=0.004$) with rupture: 42% (7/17) in type A; 55% (11/20) in type B; 64% (9/14) in type C; and 100% (15/15) in type D. Symptoms suggestive of minor leaks²⁹ before diagnosed SAH were recorded in 12 patients (29%), and minor leaks were associated with the D-type wall ($P=0.011$). Several aneurysm walls were heterogeneous with gradual change from types A or B to types C or D, mostly in the neck to fundus direction. The wall type was not associated with aneurysm size ($P>0.384$) or location ($P=0.426$) or presence of secondary pouches ($P=0.795$), but patients with B-type walls were younger (median 47 years) than patients with A-type (median 61 years) or C-type (median 58 years) walls ($P=0.021$).

Thrombosis and Fibrosis

Fresh thrombosis (Figure 1D) or OT (Figure 1C) lined the luminal aspect in 25% of unruptured and in 70% of ruptured SCAAs (Table 2). SMCs were seen more frequently in luminal OT of ruptured SCAAs (Figure 2, Table 2). OT often had areas so fibrotic that it was difficult to

TABLE 2. Patients and SCAAs

Variables	Bleeding Status		P
	Unruptured SCAAs (n=24)	Ruptured SCAAs (n=42)	
Patients			
Age	55 years (38–68)	52 years (13–76)	0.641
Gender	Males 29% (7/24)	Males 43% (18/42)	0.270
Familial background*	21% (5/24)	2% (1/42)	0.012
Patients with multiple SCAAs (≥ 2)	46% (11/24)	31% (13/42)	0.227
Patients with prior aneurysmal SAH	21% (5/24)	100% (42/42)	
Aneurysms resected for study			
No. of known ruptures	0	1 (1–4)	
Time from rupture to resection	—	18 hours (3.5 hours, 183 days) (38/42)	
Neck diameter	4 mm (2–10)	3.5 mm (2–10)	0.758
Width of fundus	6 mm (2–34)	7 mm (3–15)	0.308
Length of fundus	7.5 mm (3–29)	8 mm (3–18)	0.542
Histology of aneurysm wall			
Atherosclerotic calcifications*	21% (5/24)	5% (2/40)	0.049
Intact elastic lamina	0% (0/24)	0% (0/40)	
Remnants of elastic laminae	17% (4/24)	15% (6/40)	0.859
Endothelial lining absent*	30% (7/23)	62% (25/40)	0.014
Pads of intimal hyperplasia	42% (10/24)	48% (19/40)	0.650
Organizing thrombosis lining the wall*	21% (5/24)	60% (24/40)	0.002
Infiltrating myosin heavy chain+ cells	20% (1/5)	67% (16/24)	
Fresh thrombosis lining the wall*	17% (4/24)	20% (18/40)	0.021

Median and range are given for continuous variables.

* $P < 0.05$ (χ^2 or Mann–Whitney U test).

distinguish them from neighboring intimal hyperplasia pads, and they are collectively termed as MH/OT areas in further analysis.

Factors Associated With Rupture

Ruptured SCAA walls showed increased de-endothelialization, fresh and organizing luminal thrombosis, proliferation ratio in MH/OT areas, apoptosis ratio outside MH/OT areas, and leukocyte infiltration (CD45, CD3, CD11b, CD68, and CD163) in both areas of the wall (Table 2, Figure 3). These histological changes were not associated with minor leaks. Leukocyte density in MH/OT areas and OT were independent risk factors in logistic regression analysis ($R^2=0.46$; $P < 0.001$ for the model). Fibroblast antigen+ cells occurred equally in the walls of unruptured (39%) and ruptured (46%) SCAAs.

Leukocyte Infiltration and Cell Proliferation in the SCAA Wall After Rupture

Of the 42 ruptured SCAAs, 35 had been resected between 3.5 and 48 hours after rupture (Table 2). Proliferation ratio, T-cell density (CD3+), and macrophage density (CD163+) were increased in MH/OT areas already before 12 hours from rupture but remained stable in other areas of the wall (Figures 3 and 4). Density of CD11b+ cells and CD163+ cells in the MH/OT areas and density of CD68+ cells in other parts significantly associated with proliferation in MH/OT areas in

multiple linear regression analysis ($R^2=0.82$; $P < 0.001$ for the model).

Discussion

The cellular mechanisms of SCAA rupture have to be elucidated for development of locally delivered or systemic drug therapies. We describe in a series of 66 SCAA fundi morphological changes of the SCAA wall that correlate with rupture, association of inflammatory cell infiltration, and SMC proliferation with rupture, and association of macrophage infiltration with SMC proliferation in ruptured SCAA walls.

Morphological Changes in SCAA Wall Preceding Rupture

SCAAs tend to grow over the years.³⁰ Therefore, the SCAA wall has to undergo morphological changes that likely differ in unruptured and ruptured SCAAs. In a previous series of 27 unruptured and 44 ruptured SCAA fundi, Kataoka et al found that thick intima-like walls are mostly unruptured, and very thin and degenerated walls with hyaline deposits mostly ruptured.⁸ Other previous studies on SCAA walls describe inflammatory cells,⁹ signs of complement activation,⁹ increased protease activity,¹⁰ variations in SMC phenotype,¹¹ and apoptosis.¹²

We identified 4 histological SCAA wall types that likely reflect consecutive stages (A through D) of wall degeneration preceding to rupture. As Kataoka did, we also found

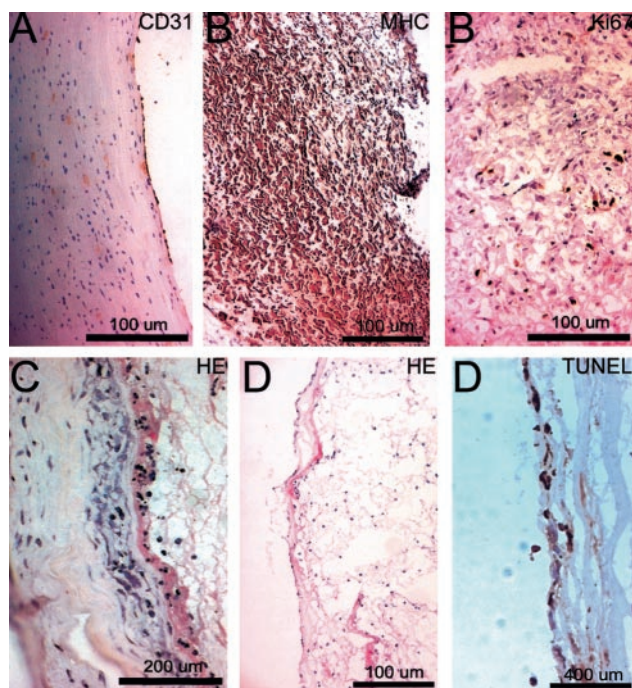


Figure 1. The 4 aneurysm wall types identified. A, Endothelialized wall with linearly organized SMCs (17/66; 42% ruptured; CD31 staining). B, Thickened wall with disorganized SMC (20/66; 55% ruptured; myosin heavy chain [MHC] and Ki67 stainings). C, Hypocellular wall with either intimal hyperplasia or organizing luminal thrombosis (14/66; 64% ruptured; HE staining). D, An extremely thin thrombosis-lined hypocellular wall (15/66; 100% ruptured; HE and TUNEL stainings).

that aneurysms with thin hyalinized walls (D-type wall in our series) were ruptured. However, in our series, also as many as 55% (11/20) of thick intima-like walls (B-type) had ruptured. This may reflect differences in Finnish and Japanese SCAA populations. Interestingly, in our series, B-type (thick intima-like) walls occurred in younger patients than A-type (organized) or C-type (hypocellular with luminal thrombosis) walls. Possible association between age and SCAA wall maintenance and repair capacity warrants further studies.

Maintenance and Repair of SCAA Wall

The wall of unruptured SCAAs may remain intact for years.³⁰ Thus, strong maintenance and repair mechanisms are mandatory. Our results suggest that before rupture, the SCAA wall becomes unstable and undergoes morphological changes that start at an undefined time interval before rupture. These changes reflect the effect of risk factors that predispose to rupture as well as the maintenance and repair mechanisms trying to prevent rupture. The factors distinguishing unruptured and ruptured SCAAs in our series were: decellularization, apoptosis, and degeneration of wall matrix; de-endothelialization; thrombus organization; proliferation; and inflammatory infiltration. Most of these are features related to MH (ie, the mechanism of how generally the arterial wall responds to injury or hemodynamic stress).^{16–18} In MH, proliferation and migration of vascular SMCs lead to formation of a thickened fibroid

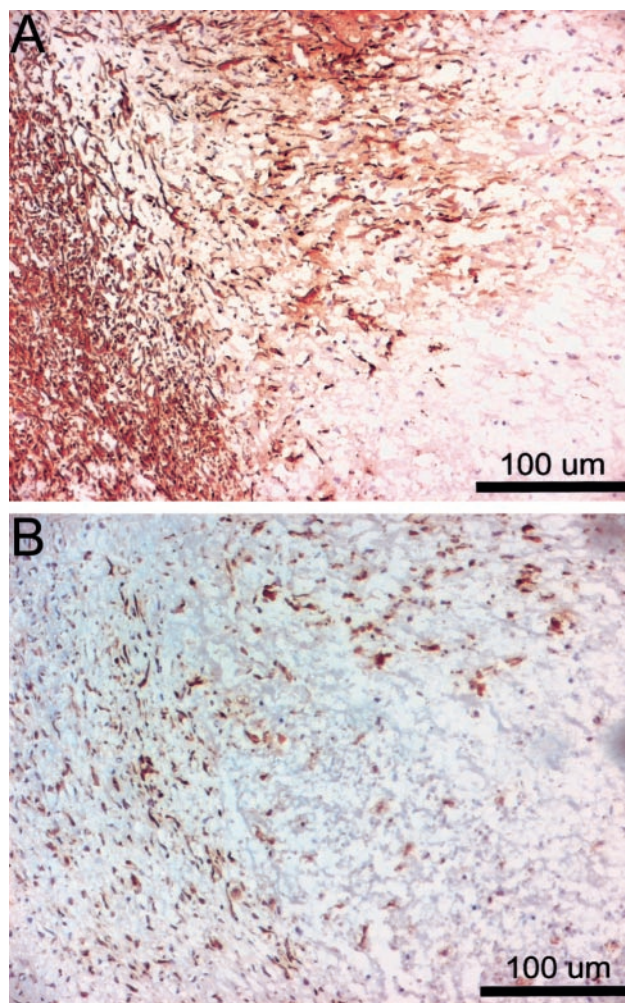


Figure 2. Migration of SMC from the aneurysm wall to a luminal thrombus. A, Immunostaining for myosin heavy chain. B, Immunostaining for fibroblast-antigen. The microphotographs were taken under $\times 10$ magnification from consecutive sections of a twice-ruptured right Acoma aneurysm (11 \times 6 mm) in a 28-year-old male. Time from last rupture 14 hours.

layer on the luminal surface of the vessel.^{16–18} During MH formation, the SMCs that migrate from the vascular wall to the luminal surface secrete matrix metalloproteinases that destroy parts of the wall matrix and make the migration of SMCs possible.³¹ The morphological changes that result from the MH and matrix destruction are collectively referred to as remodeling of the vascular wall.^{16,17} Although MH is an adaptation mechanism of arteries to hemodynamic stress, in SAH patients, for undefined reasons, vascular wall remodeling was insufficient to prevent SCAA rupture. Paradoxically, in SCAAs, remodeling might even facilitate rupture because of increased matrix proteolysis.³¹ It would be important to study aneurysms at a few weeks after rupture, but in our series, all but 6 aneurysms were clipped within 48 hours.

Inflammation in SCAA Wall

Ruptured SCAA walls show inflammation.⁹ It is not known whether inflammation triggers the rupture of the SCAA wall

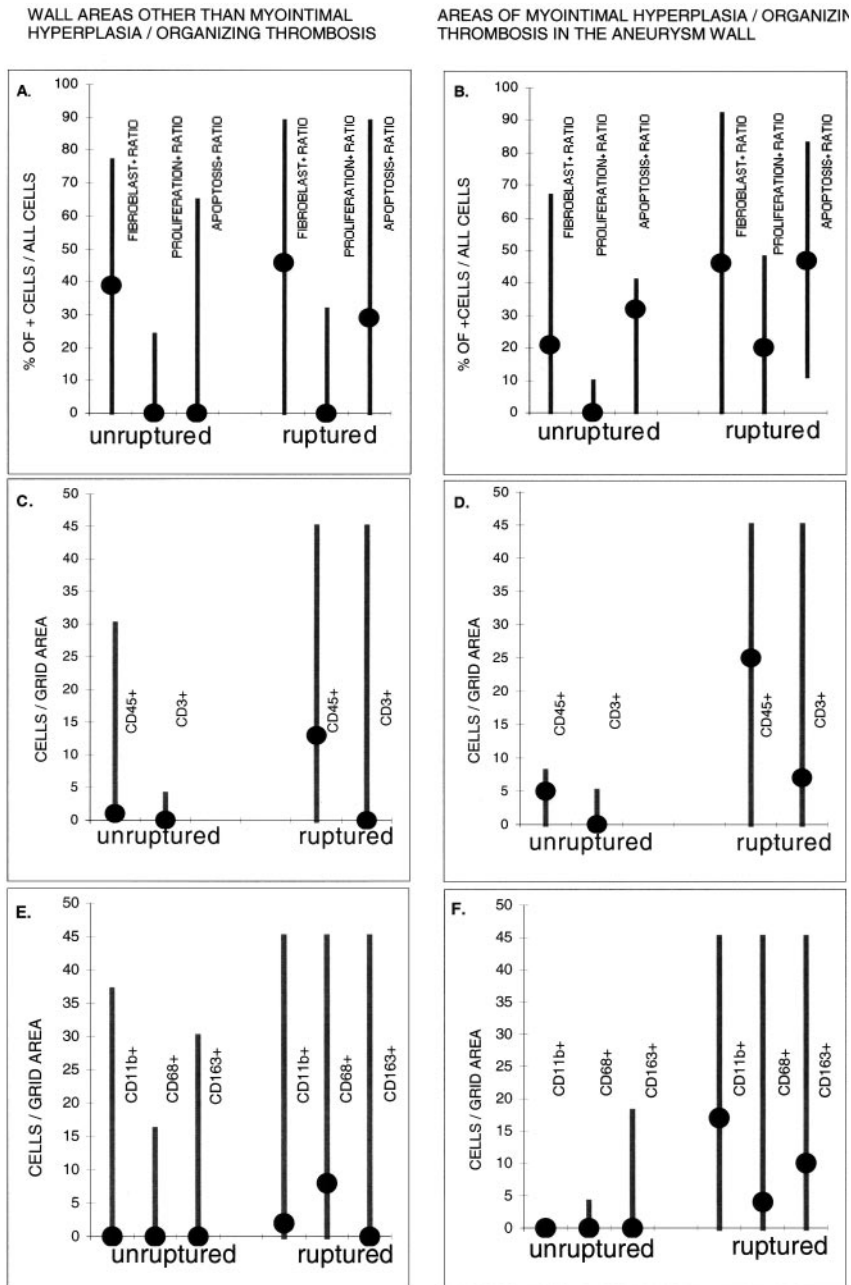


Figure 3. The ratios of fibroblast-antigen+, proliferating and apoptotic cells from total cell number, and the density of inflammatory cells in the wall of unruptured and ruptured aneurysms. Median values and range are given for areas of MH/OT and other areas of the aneurysm wall. Significant differences ($P < 0.05$; Mann-Whitney U test) between unruptured and ruptured aneurysm were found in: (1) the proliferation ratio in MH/OT areas, (2) the ratio of apoptosis in wall areas other than MH/OT, (3) the density of all inflammatory cell types in areas of MH/OT, and (4) the density of CD45+, CD163+, and CD11b+ cells also in other areas.

causing SAH. However, it is known that infiltrating leukocytes, mainly T-cells and macrophages, stimulate SMC proliferation in areas of vascular wall thickening.¹⁸ We found that T-cell and macrophage infiltration associate with rupture, and furthermore, macrophage infiltration associates with SMC proliferation in the SCAA wall. Therefore, we hypothesize that in the SCAA wall, macrophages may stimulate SMCs to change phenotype and proliferate, thus promoting fibrosis. That SMC proliferation and T-cell and macrophage infiltration were increased in samples resected <12 hours from rupture suggests that these changes were, to some extent, present before rupture because in healthy arterial wall, they occur in response to injury during the first 24 hours or later (T-cell and macrophage infiltration as well as SMC proliferation).^{17,32}

Therapeutic Implications

Only few diagnosed SCAAs will occlude spontaneously.³⁰ It is not known why luminal thrombosis, SMC migration, and vascular wall remodeling fail to prevent rupture and occlude untreated SCAA pouches. Systemic or locally delivered selected agents that stimulate SMC proliferation and migration to luminal thrombus might promote the occlusion. Our data suggest that inflammatory cell infiltration and SMC proliferation increase in the SCAA wall before rupture, and we hypothesize that they are part of the adaptation and repair mechanism of the SCAA wall. Locally delivered selected proinflammatory agents stimulating SMC proliferation and matrix synthesis might reinforce the SCAA wall. In addition, matrix metalloproteinase inhibitors that reduce proteolysis in mechanical arterial

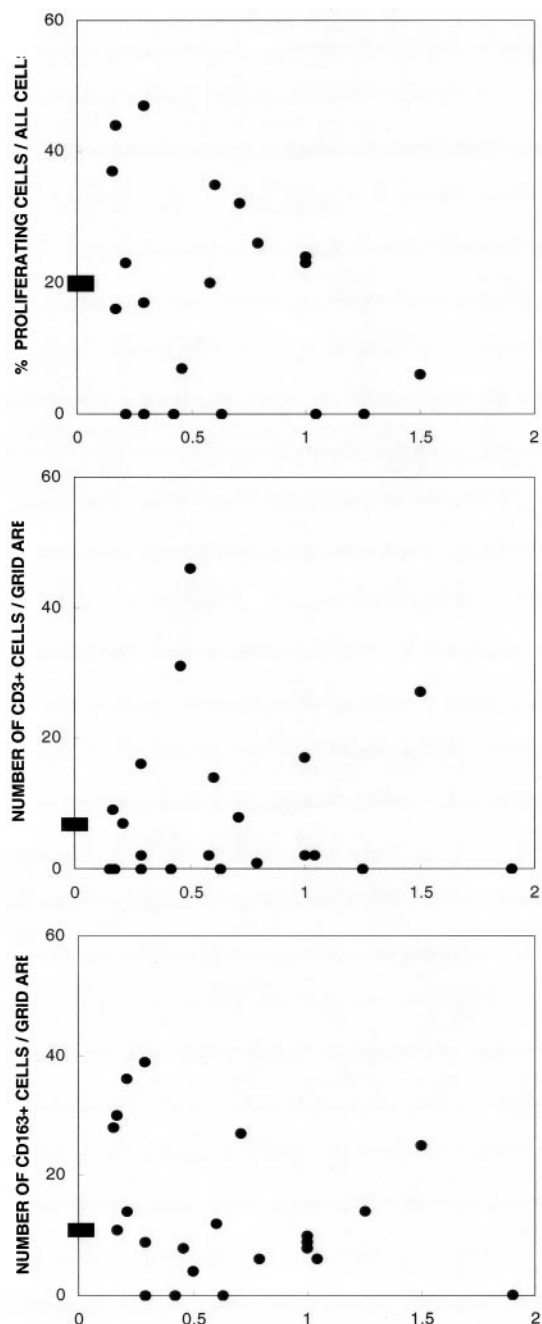


Figure 4. Proliferation ratio, T-cell (CD3+) density, and macrophage (CD163+) density in areas of MH/OT in function of time from rupture. Median values are marked on the y-axis with filled boxes.

wall injury models^{33,34} might inhibit harmful matrix degradation in the SCAA wall and prevent rupture.

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