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Assessment of Endothelialization of Aneurysm Wall Over Time in a Rabbit Model Through CD31 Scoring

Praveen Kolumam Parameswaran, MBBS¹, Daying Dai, MD, PhD¹, Yong Hong Ding, MD¹, Tina Gunderson, MS², David F Kallmes, MD¹, and Ramanathan Kadirvel, PhD¹

¹Department of Radiology, Mayo Clinic, Rochester

²Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester

Abstract

Background—Intracranial aneurysms represent a significant health concern and are poorly understood despite decades of research. Our study focused on understanding temporal patterns of endothelial cell distribution in different spatial locations within the aneurysm early after creation in a rabbit model.

Methods—Elastase induced saccular aneurysms were created in rabbits and harvested at day 1 (n=3) and 2 (n=5), 4 (n=4), 8 (n=5), and 12 (n=6) weeks. Sham operated controls (n=3) were harvested on the same day. Aneurysm and control tissue samples were subjected to *en face* whole-mount CD31 staining for endothelial cells. Semiquantitative scoring was performed on the basis of endothelial coverage of the vessel wall (proximal, middle, and distal portions of the aneurysm dome). Mixed-effects models were used to assess the effect of time and aneurysm section on endothelial coverage.

Results—Aneurysmal segments were near completely deendothelialized at 4 and 8 weeks but had reendothelialized by 12 weeks. Compared to control, aneurysms at all the time points showed decreased endothelialization, but the difference was only significant compared 4- and 8-week groups. Both time ($P=.03$) and aneurysm section ($P=.07$) were significantly associated with degree of endothelialization. Proximal locations showed increased endothelialization compared with distal locations ($P=.03$).

Conclusion—In experimental aneurysms of rabbits, endothelial cells regress during the first month after creation, followed by ascending reendothelialization that stays incomplete. These findings suggest that repopulation of endothelial cells comes from resident cells in the adjacent

Corresponding author: Daying Dai, MD, PhD, Department of Radiology, Mayo Clinic, 200 First St SW, Rochester, MN 55905, Dai.Daying@mayo.edu, 507-266-3350, 507-255-7872 (fax).

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COMPETING INTEREST STATEMENT

None.

AUTHOR CONTRIBUTORSHIP STATEMENT

PKP and DD contributed to tissue processing, slides staining, interpretation of data, and drafting of the manuscript.

YHD contributed to the aneurysm model creation.

TG contributed to statistics of the study.

DFK and KR contributed to the conception and design of the study and to revision of the article critically for important intellectual content.

parent artery and that deranged hemodynamics may affect full reconstitution of endothelial cells long term.

Keywords

Intracranial aneurysms; CD31 staining; endothelial distribution; endothelial pattern; endothelial score; rabbit model

INTRODUCTION

Intracranial aneurysms (ICA), prevalent in 3% to 4% of the population, are a relatively common vascular disorder¹². ICAs have varied wall structure, cell content, and clinical outcome. The mechanism in healing an aneurysm continues to be enigmatic, and despite treatment with endovascular devices, the recurrence rates are still high (20%–25%)³⁴⁵. Studies have shown that endothelialization forms the critical component in arterial hemostasis and in achievement of complete occlusion of the aneurysm neck⁶⁷. Moreover, incomplete endothelialization at the aneurysm neck has been accounted for the increase in recurrences following coil embolization⁸⁹. Therefore, it is essential to understand the pattern of distribution of endothelial cells over time after the formation of aneurysm. This paper is an attempt to fill the gaps in knowledge about aneurysm healing and explain the reason behind incomplete endothelialization noted after treatment with endovascular devices.

Since the wall represents the primary pathologic factor indeed, the actual site of enlargement and rupture we believe that a comprehensive exploration of aneurysm wall can provide valuable insight that improves patient treatment. Several studies have shown CD31 as a reliable marker for identification of endothelial cells, and hence scoring based on CD31 expression is a better way to understand the distribution of endothelial cells¹⁰¹¹.

The elastase-induced aneurysm model of the rabbit has been used as an established clinical model for studying aneurysms and has a hemodynamic status comparable to human aneurysms^{12–15}. Several studies using the rabbit model have demonstrated the shear stress patterns and device testing of the aneurysms^{14–16}. However, no study has highlighted the distribution pattern of endothelial cells over time after aneurysm formation. In our study, we attempted to characterize the pattern of deendothelialization and reendothelialization of vessel wall over time at different spatial locations in the aneurysm in our rabbit model.

METHODS

Aneurysm Creation

The Mayo Clinic Institutional Animal Care and Use Committee approved all protocols of the study. Elastase-induced saccular aneurysms were created in 26 New Zealand white rabbits and aneurysm tissues were harvested at day 1 (n=3) and 2 (n=5), 4 (n=4), 8 (n=5), and 12 (n=6) weeks. In the control group (n=3, day 0), similar procedures of aneurysm creation was followed but instead of the elastase, saline incubation was done in RCCA. Detailed procedures for aneurysm creation have been described elsewhere¹⁷¹⁸¹⁹. In brief, rabbits (3–4 kg) were induced with intramuscular injection of ketamine and xylazine, (35 and 5 mg/kg, respectively) and maintained with 1–3% isoflurane. Using sterile technique,

the right common carotid artery (RCCA) was exposed and ligated distally. A 5F sheath (Cordis Endovascular, Miami Lakes, Fla) was advanced retrograde in the RCCA to a point approximately 3 cm cephalad to the origin of RCCA. A roadmap image was obtained by injection of contrast through the sheath retrograde in the RCCA, to identify the junction between the RCCA and the subclavian and brachiocephalic arteries (Advantx; General Electric, Milwaukee, Wis). Through the indwelling sheath, a 3F Fogarty balloon (Baxter Healthcare Corporation, Irvine, Calif) was advanced to the origin of the RCCA at its junction with the right subclavian artery. The balloon was inflated with just enough iodinated contrast material to achieve flow arrest in the RCCA. Porcine elastase (5.23 U/mgP, 40.1 mgP/mL, approximately 200 U/mL; Worthington Biochemical, Lakewood, NJ) mixed with iodinated contrast material was incubated in the dead space of the RCCA, above the inflated balloon, through a microcatheter (Tracker 10; Target Therapeutics, Fremont, Calif). Balloon position and shape were documented with the use of fluoroscopic spot imaging, which was saved and printed. After incubation of the elastase solution, the balloon and sheath were removed, and the RCCA was ligated below the sheath entry site. The harvested aneurysm tissue was dissected along the midline into dorsal and ventral pieces. The dorsal piece was processed for whole-mount en face immunostaining with CD31 for endothelial cells.

Whole-mount en face immunostaining

The freshly harvested dorsal piece of aneurysm wall was pinned to a dish coated with Sylgard (Dow Corning Corp) to expose its lumen side and was completely immersed in 10% neutral buffered formalin to fix for 2 hours at room temperature (RT). The sample was washed with TBS for 15 minutes in 3 washes. It was then blocked with 5% donkey serum in TBST for 1 hour at RT, followed by incubation with primary antibody (CD31, 1:20–30 in 0.3% Triton X-100 [Sigma-Aldrich Co, LLC] in TBS) for 1 hour at RT, overnight at 4°C. The samples were washed again with TBS and incubated with secondary antibody (Cy3 conjugated donkey antimouse IgG, 1:200 in TBS) for 2.5 hours at RT and counterstained with Sytox green (ThermoFisher Scientific) (1:1000). Finally, the sample was scoped with confocal laser microscope.

Each sample was divided into 3 portions for score: distal (apical one-third far away from the parent artery), proximal (basal one-third close to the aneurysm neck), and middle (between proximal and distal areas). Five fields from each portion were randomly selected for evaluation to achieve 5 scores for each portion.

Scoring of endothelial cells are performed as stated below (Figure 1)

- 0 -- Complete lack of coverage of CD31 positive endothelial cells
- 1-- Scattered, sparse cells positive for CD31, covered the lumen side of the wall
- 2 -- Small, isolated, patchy, cell clusters are positive for CD31
- 3 -- The cell clusters which are positive for CD31 are larger than that seen in 2
- 4 -- Majority of lumen side of the wall is covered with CD31 + cells, with the exception of some small gaps among cells

5 -- The lumen side of the wall is completely covered with CD31 + cells.

Statistical methods

Each rabbit had 5 fields scored from each aneurysm portion (proximal, middle, and distal). Scoring consistency between fields was tested using Krippendorff's α . Field scores were averaged to create a composite score for each aneurysm location within the study rabbit. A mixed-effects model was used to test for association of score with time and location as shown Table 1. Denominator degrees of freedom were corrected using the Kenward-Rogers method. Model diagnostics were performed. Overall variable significance in the model and model-based means are presented. Because of the preliminary nature of the investigations, significance was set at $\alpha=0.10$. Analyses were performed with statistical software (SAS Institute Inc).

RESULTS

Aneurysms harvested at day 1 were excluded from the analysis due to the presence of fresh, unorganized thrombus within the dome, which prevented us from processing the samples for en face CD31 immunostaining.

Sham operated control samples showed the highest level of endothelialization (Figure 2), but the difference was only significant compared to the 4- and 8-week groups (Table 2). Compared to the control group, the groups at 4 weeks and 8 weeks showed decreased endothelialization (-2.18 [90% CI $-3.39, 0.974$], $p=0.006$; -1.65 [90% CI $-2.81, -0.497$], $p=0.023$). Of the remaining comparisons, the 2-week and 12-week groups showed significantly greater endothelialization than the 4-week group (2 week – 4 week: 1.53 [90% CI $0.468, 2.59$], $p=0.022$; 2 week – 12 week -1.34 [$-2.36, -0.317$], $p=0.036$); none of the other pairwise comparisons between different times of sacrifice were significantly different (Table 2). Thus our results show a trend of decrease in CD31 scores starting from day 0 to 4 weeks followed by increase in CD 31 scores at 8 and 12 weeks. However the scores at 12 weeks does not match the scores of control (day 0).

The coefficient of reliability among the five raw CD31 positive scores at each animal $\times$ location combination was $\alpha=0.755$. Descriptive statistics for the composite scores per rabbit were calculated and Figure 2 shows the box and whisker plot for composite CD31 scores at each time point and aneurysm section location.

Group mean estimates and confidence intervals (CI) from the linear mixed model are shown in Table 1. Both location and time of sacrifice were significant at the $p=0.1$ level (location $p=0.078$, time $p=0.032$). The interaction effect (time $\times$ location) was not significant and was dropped from the model. Both middle and proximal locations had higher endothelialization when compared to distal, though only proximal was significantly different (mean difference/ [90% CI] 0.417 [$-0.027, 0.862$], $p=0.12$; 0.600 [$0.16, 1.04$], $p=0.028$ respectively). Middle locations were also decreased compared to proximal, but this difference was not significant (-0.183 [$-0.627, 0.262$], $p=0.49$) (Table 2).

DISCUSSION

In our study, a moderate deendothelialization was noted in the sham operated control in the aneurysm wall. It was followed by further deendothelialization at 2 and 4 weeks and reendothelialization at 8 and 12 weeks. Endothelialization was not complete near the 12-week point, probably because of deranged hemodynamics that altered the blood flow within the aneurysm.

Aneurysm formation occurs because of weakening in the vessel wall due to stripping of the internal elastic lamina and tunica media, resulting in an outpouching of only tunica intima and adventitia. It was conventionally believed that endothelial dysfunction leading to denudation occurs during aneurysm formation, followed by reendothelialization and collagen deposition²⁰. In our rabbit aneurysm model, we observed initial denudation of endothelial cells associated with advancement of sheath and balloon inflation during aneurysm creation. We were not able to clearly delineate the endothelial injury attributed to the elastase injection during aneurysm creation owing to the unorganized thrombus formation within the aneurysm 24 hours after creation. However, we see a pattern of early deendothelialization at aneurysm creation and further deendothelialization at 2 and 4 weeks could be attributed to the altered flow pattern within the aneurysm.. This finding is clinically relevant because deendothelialization and hypocellular vessel wall are important contributors in aneurysm rupture, as reported by Frosen et al²¹. We believe the wall is at increased risk of rupture around 4 weeks after aneurysm creation. Our rabbit model has histologic characteristics identical to human aneurysm²². This finding also sheds light on the reason for the rupture of small aneurysms in certain patients²²⁻²⁴

The current knowledge of endothelialization pertaining to aneurysm healing has been shown in relation to growth of cells on endovascular coils and flow diverter stents^{15,25}. The source of endothelialization after endovascular treatment continues to be controversial. Dai et al²⁶ have shown that initial endothelialization following coil embolization occurs as a result of continuation of cells from the parent artery. Liu et al²⁷ have shown that endothelial progenitor cells in circulation contribute to the endothelialization following coil embolization²⁸⁻³⁰. On the basis of our initial endothelialization noted till 2 weeks of aneurysm creation, we believe that endothelial cells remain contiguous with the parent artery. Following this we observed further deendothelialization at 4 weeks. We believe this further denudation of endothelial cells may be due to altered flow pattern within the aneurysm. Overtime, the reendothelialization occurs, with the new flow pattern starting from the aneurysm neck, and reaches the dome around 12 weeks, as reported by findings in our study. This finding further confirms our theory of endothelial cell origin from the parent artery because reendothelialization begins in the neck close to the parent artery at 8 weeks, whereas no endothelial cells were noted at the dome in that timeframe. Moreover, endothelialization was noted to be greatest at baseline (day 0) of aneurysm formation and did not have a similar cell number around 12 weeks (Figure 2). This outcome might be due to the altered hemodynamics within the aneurysm leading to incomplete endothelialization, subsequently leading to recanalization of aneurysms.

Currently, no study delineates the pattern of distribution of endothelial cells in the aneurysm wall and no scoring system is available to quantify endothelialization across different time points. Our scoring system not only quantitates spatial distribution of endothelial cells in aneurysms but also can help predict the rate of healing of aneurysms after treatment with endovascular devices. This information will enable us to identify devices that promote better healing of aneurysms. Future studies are warranted to explore new options in stabilizing the endothelial cells during the first month of aneurysm formation that might lead to better outcomes in aneurysm treatment.

LIMITATIONS

Our study has limitations. The aneurysms produced in the elastase-induced rabbit model continue to be stable after 1 month and do not enlarge and rupture. Thus, the endothelialization in enlarging ruptured aneurysms cannot be correlated in our model. Even though vascular smooth muscle cells are also critical in aneurysm formation, owing to technical difficulty of scoring smooth muscle cells, we only analyzed endothelialization patterns. The presence of unorganized thrombus in the aneurysm sac 24 hours after aneurysm creation limiting us to understand the role played by elastase in the deendothelialization in our model. With regards to the role played by altered flow pattern within the aneurysm we can only postulate and not definitively state that deranged hemodynamics is responsible for the stripping of endothelial cells observed at 4 and 8 weeks following aneurysm creation.

CONCLUSION

In experimental aneurysms of rabbits, endothelial cells regress in all regions during the first month after creation, followed by ascending reendothelialization that remains incomplete. These findings suggest that repopulation of endothelial cells comes from resident cells in the adjacent parent artery and that deranged hemodynamics may affect the full reconstitution of endothelial cells long term.

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Figure 1. CD31 stained endothelial cells categorized into five scores based on the distribution of endothelial cells. Score of 5=highly confluent endothelial cells; score 0=no endothelial cells.

Figure 2.

Comparison of CD31 scores and time points of sacrifice at different aneurysm section locations. Each point represents the composite score for one rabbit. The center line on each box represents the median; the limits of the box, and the interquartile range. Endothelialization is greatest in the control group (mean 2.47) followed by de-endothelialization in week 2 (mean 1.81) and week 4 (mean 0.283). Re-endothelialization is noted again at week 8 in the proximal and middle portions of the aneurysms. Re-endothelialization is noted in all three regions at week 12 (mean 1.62).

Table 1

Estimate of Group Means From the Mixed-Effects Model.

Effect	Value	Mean	90% CI Upper	90% CI Lower	P value ^a
Location	Distal	1.06	0.643	1.48	0.07
	Middle	1.48	1.06	1.9	
	Proximal	1.66	1.24	2.08	
Time	Controls	2.47	1.55	3.38	0.05
	2 weeks	1.81	1.11	2.52	
	4 weeks	0.283	-0.508	1.07	
	8 weeks	0.813	0.105	1.52	
	12 weeks	1.62	0.976	2.27	

^a Represents variable in the overall mixed-effects model.

Table 2

Mixed-Model Pairwise Comparisons Between Levels of Each Variable

Effect	Comparison	Estimate	90% CI Upper	90% CI Lower	p
Location	Middle vs Proximal	-0.183	-0.627	-0.262	0.493
	Middle vs Distal	0.417	-0.027	0.862	0.122
	Proximal vs Distal	0.6	0.156	1.04	0.028
Time	Week 2 vs Control	-0.653	-1.81	0.503	0.340
	Week 4 vs Control	-2.18	-3.39	-0.974	0.006
	Week 8 vs Control	-1.65	-2.81	-0.497	0.023
	Week 12 vs Control	-0.844	-1.96	0.275	0.207
	Week 2 vs Week 4	1.53	0.468	2.59	0.022
	Week 2 vs Week 8	1	-0.001	2	0.100
	Week 4 vs Week 8	-0.53	-1.59	-0.532	0.398
	Week 2 vs Week 12	0.191	-0.768	1.15	0.734
	Week 4 vs Week 12	-1.34	-2.36	0.317	0.036
	Week 8 vs Week 12	-0.809	-1.77	0.15	0.161