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Loss and Apoptosis of Smooth Muscle Cells in Intracranial Aneurysms

Studies with in situ DNA End Labeling and Antibody Against Single-Stranded DNA

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Summary

Pathological specimens were collected from 14 unruptured and 13 ruptured aneurysms at the time of clipping and studied in order to assess the underlying mechanism of rupture by investigating degeneration of the aneurysmal wall and possible involvement of apoptosis. Immunohistochemistry with anti-actin antibody showed few smooth muscle cells in the ruptured aneurysms and replacement of the muscularis layer by a fibro-hyalin tissue. However, at least one layer of smooth muscle cells was clearly observed in the unruptured aneurysms. Thus, smooth muscle cells in the wall of the ruptured aneurysms were much more degenerated than those in the wall of unruptured aneurysms. In addition, unruptured aneurysms with an angiographically smooth wall showed well-layered positive staining for anti-smooth muscle actin antibody while those with irregular shapes rarely reacted. We found, for the first time, evidence of DNA fragmentation in the aneurysmal wall. Apoptotic bodies were detected by means of a terminal transferase (TdT)-mediated dUTP biotin nick end labelling technique (TUNEL) and an anti-single-stranded DNA antibody in 54% (7/13) of the ruptured aneurysms. In contrast, apoptotic bodies were found in only 7% (1/14) of the unruptured cases. These results suggest that apoptotic cell death might be involved in the rupture of aneurysms.

Keywords: Aneurysm; anti-single-stranded DNA antibody; apoptosis; DNA fragmentation.

Introduction

Recent advances in diagnostic modalities have led to more frequent identification of unruptured aneurysms. A major problem, however, is whether to treat them or not. Natural history as well as surgical mortality and morbidity should all be taken into consideration when evaluating the advisability of surgery for unruptured aneurysms. Studies to identify the underlying mechanism in aneurysmal rupture are therefore essential. To begin with haemodynamic stress can be assumed to be one of the main factors in the rupture of aneurysms. A very thin locus in the wall, where the normal vascular structure is lost, is often seen during surgery of unruptured aneurysms. Such a fragile site might well be the locus for rupture, but it is still not clear how the aneurysmal wall becomes fragile. Recent evidence has shown that apoptosis is involved not only in the normal developmental phase but also in many pathological situations [12]. Apoptosis is referred to as silent cell death in contrast to necrosis, because it is not accompanied by an inflammatory reaction. Apoptosis might thus be present but undetected in the wall of an aneurysm and reduce the strength of the wall.

With this in mind, we studied pathological specimens obtained at aneurysm surgery to assess the underlying mechanism of rupture. We also studied degeneration of the aneurysmal wall and the possible involvement of apoptosis.

Patients and Methods

Between January and December 1993, we collected pathological specimens from 27 aneurysms at surgery. After the aneurysms were clipped by a standard microsurgical technique, their domes were resected if the surgeons felt it was safe to do so. Fourteen of the aneurysms were unruptured and 13 ruptured. We divided the unruptured aneurysms further into two types based on the angiographic findings: smooth (round and oval shapes) and irregular (conical and uneven shapes) (Fig. 1). The angiograms were evaluated independently by two assistants without evidence of any inter-observer differences.

The pathological samples were fixed overnight in 75% ethanol, dehydrated, embedded in paraffin, and cut into serial sections 10 μm thick.

Immunohistochemical Study with Anti-Actin Antibody

After deparaffinisation, the sections were reacted with anti-smooth muscle actin antibody and processed with the avidin biotin peroxidase-complex (ABC) method. In brief, they were incubated

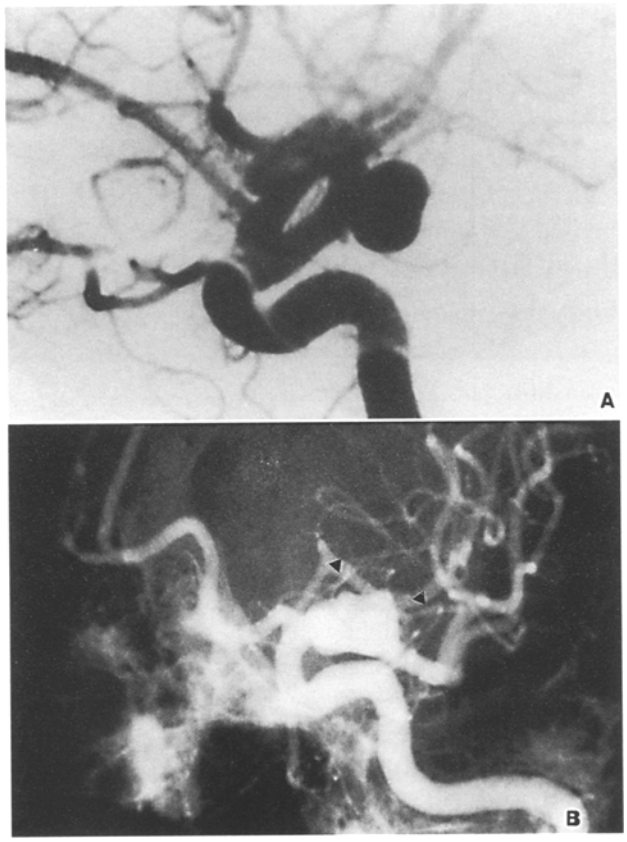

Fig. 1. Typical angiograms of internal carotid aneurysms. (A) Smooth type (round and oval shape). (B) Irregular type (conical and uneven shape)

for 15 min in methanol containing 1% H_2O_2 at room temperature (RT) to block endogenous peroxidase activity. Sections were then rinsed three times with 0.05 M tris buffered saline (ph 7.6), incubated for 30 min in 2% normal horse serum, tipped off, and reacted overnight at 4° C with anti-smooth muscle actin monoclonal antibody (MA-931, ENZO Biochem Co., NY, USA) at 1:2000 dilution. For the next step, biotinylated horse anti-mouse IgG and then an avidin-biotin-peroxidase-complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) were applied for 30 min each at RT. Immunolabelled peroxidase was detected with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. Finally, sections were lightly counterstained with hematoxylin, dehydrated, coverslipped, and observed under a light microscope.

In situ Detection of DNA Fragmentation

We used a molecular biological-histochemical system (ApopTag kit; Oncor, Gaithersburg, MD, USA) for sensitive and specific detection of DNA fragmentation [2] in the aneurysmal wall. The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3′-OH ends of DNA (TUNEL) and the ensuing in situ synthesis of a poly-deoxynucleotide polymer. The labelling targets are the new 3′-OH DNA ends generated by DNA fragmentation, which are typically localized in morphologically identifiable nuclei and apoptotic bodies. Cells containing apoptotic bodies are referred to as "apoptotic cells" [2, 17, 18]. The procedure was performed in accordance with the manufacturer's instructions. In brief, the sections were first digested by proteinase K after deparaffinisation. Endogenous peroxidase activity was quenched with 2% H_2O_2 in phosphate-buffered saline (PBS) for 5 min followed by rinsing in PBS. Next, an equilibration buffer was applied directly to the section, followed by the application of working-strength TdT enzyme. Sections were incubated in a humid chamber for one hour at 37° C and then placed in a stop/wash buffer for 30 min at 37° C. After three washings in PBS, peroxidase conjugated anti-digoxigenin-antibody was applied to the slides for 30 min at 37° C, and the incorporated peroxidase activity detected with DAB and H_2O_2. For negative controls, adjacent sections were processed in the same way but distilled water was substituted for the TdT enzyme in the preparation of working-strength TdT.

We also used an antibody specific for single-stranded DNA to identify the cells exhibiting DNA fragmentation. The rabbit polyclonal antibody raised against single-stranded DNA was kindly donated by Dr. Sugiyama (Dept. of Biochemistry, Akita University, Akita, Japan) [4, 6, 10, 11, 19]. In brief, sections were incubated for 20 min in methanol containing 1% H_2O_2 at RT to block the endogenous peroxidase activity after deparaffinisation. They were then rinsed three times with PBS, and incubated for 30 min in 10% normal goat serum. Next, the anti-single-stranded DNA polyclonal antibody was applied at a dilution of 1:5000 and incubated overnight at 4° C. After reaction with biotinylated goat anti-rabbit IgG, the avidin-biotin-peroxidase-complex was applied for 30 min at RT. Subsequent steps were identical to those described above. The χ^2 test was used for statistical analysis.

Results

Immunohistochemistry for Smooth Muscle Cells

The immunohistochemical technique for smooth muscle actin was used for better visualisation of the degeneration of smooth muscle cells in the aneurysmal dome. In the ruptured aneurysms, few smooth muscle cells were seen, and these stained sparsely with anti-smooth muscle actin antibody. The muscularis layer had been replaced by a fibro-hyalin tissue and the inner elastic layer had disappeared (Fig. 2 A). In the unruptured aneurysms, in contrast, well-layered positive staining for anti-smooth muscle actin antibody was oberved in nine aneurysms (Fig. 2 B). Although the rest of the unruptured aneurysms showed sparse reactivity as in the ruptured cases, at least one layer of smooth muscle cells was clearly observed in the unruptured aneurysms.

Our next aim was to establish whether any relation existed between the angiographic findings and the anti-actin immunoreactivity. Six of the unruptured aneurysms showed a smooth wall on the angiogram and eight an irregular shape. In the smooth wall group, none of the aneurysms showed negative staining for the anti-actin antibody. In contrast, five of the irregularly shaped aneurysms showed reactivity,

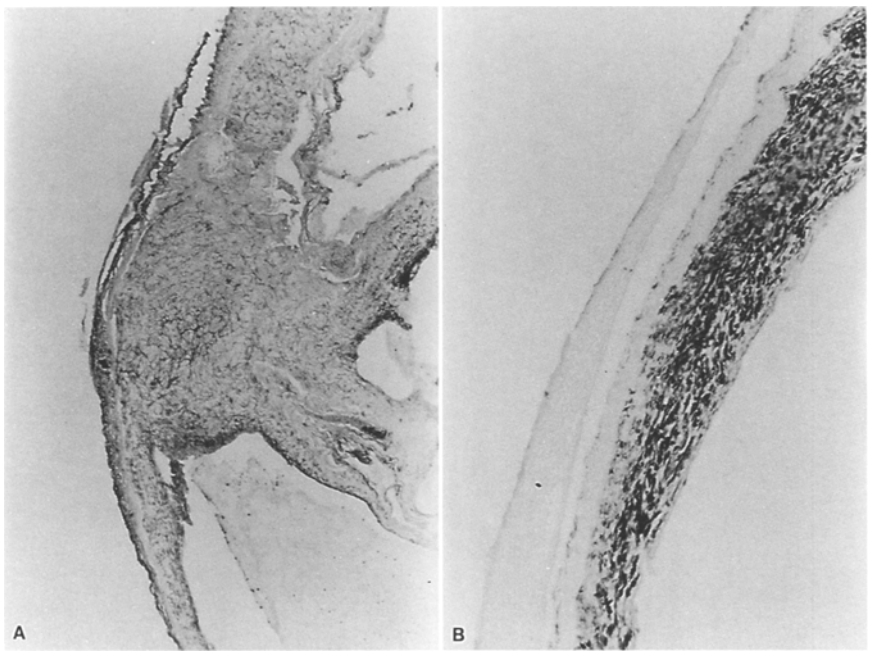

Fig. 2. Immunohistochemical study with anti-smooth muscle actin antibody. (A) shows severe degeneration of smooth muscle cells in the media of a ruptured aneurysm. Smooth muscle cells were rarely seen and the muscularis layer had been replaced by a fibro-hyalin tissue. (B) shows a representative photo of an unruptured case with smooth aneurysm, which is positive for anti-smooth muscle actin antibody and shows well-preserved smooth muscle cells in the media of the wall. (Original magnification; X200)

Table 1. *Relationship between the Radiology and Pathology in Unruptured Aneurysms*

Wall type	Immunohistochemistry of anti-smooth muscle antibody	
staining	Sparse or no reactivity	Well-layered positive
Smooth	0% (0/6)	100% (6/6)[a]
Irregular	62.5% (5/8)	37.5% (3/8)

[a] $P<0.05$ vs irregular wall aneurysm by χ^2 test

although sparse, for anti-smooth muscle actin. The χ^2 test revealed that the smooth wall group was significantly positive for anti-actin immunoreactivity, suggesting well-preserved smooth muscle cells (Table 1).

Examination for DNA Fragmentation

Examination for DNA fragmentation with the TUNEL method was performed on all samples. The light microscopic identification of DNA fragmentation depended upon the recognition of round or oval bodies (apoptotic bodies) (Fig. 3 A, 3 D), that belonged to cells in the lamina muscularis of the aneurysm (Fig. 3 F). The earliest recognizable stage in DNA fragmentation is chromatin condensation around the margin of the nucleus forming either crescent caps (Fig. 3 B, 3 E) or rings. Normal nuclei, which might have insignificant numbers of DNA 3′-OH ends, do not stain with either the TUNEL or anti-single-stranded DNA antibody. Necrotic cells, which in some instances might contain stainable concentrations of DNA ends, can be stained with the TUNEL. Therefore, they should be differentiated from apoptotic cells on the basis of their staining pattern. Furthermore, the staining in necrotic cells appears more diffuse than in apoptotic cells [9]. Figure 3 C shows an example of necrotic cells and associated neutrophils migrating across the endothelial cells. They could be distinguished from apoptotic cells by their diffuse, light brown color and lack of apoptotic bodies. With the anti-single-stranded DNA antibody, no positive staining of necrotic cells occured, making it easy to evaluate the results. The apoptotic cells were located in the media of the wall and found predominantly in the periphery of the ruptured dome.

The results of light microscopic examination of in

Table 2. *DNA Fragmentation Detected with the TUNEL and Anti-Single-Strand DNA Staining*

Aneurysm	DNA fragmentation	
	−	+
Rupture	46% (6/13)	54% (7/13)[a]
Unrupture	93% (13/14)	7% (1/14)

[a] $P<0.01$ vs unruptured aneurysm by χ^2 test.

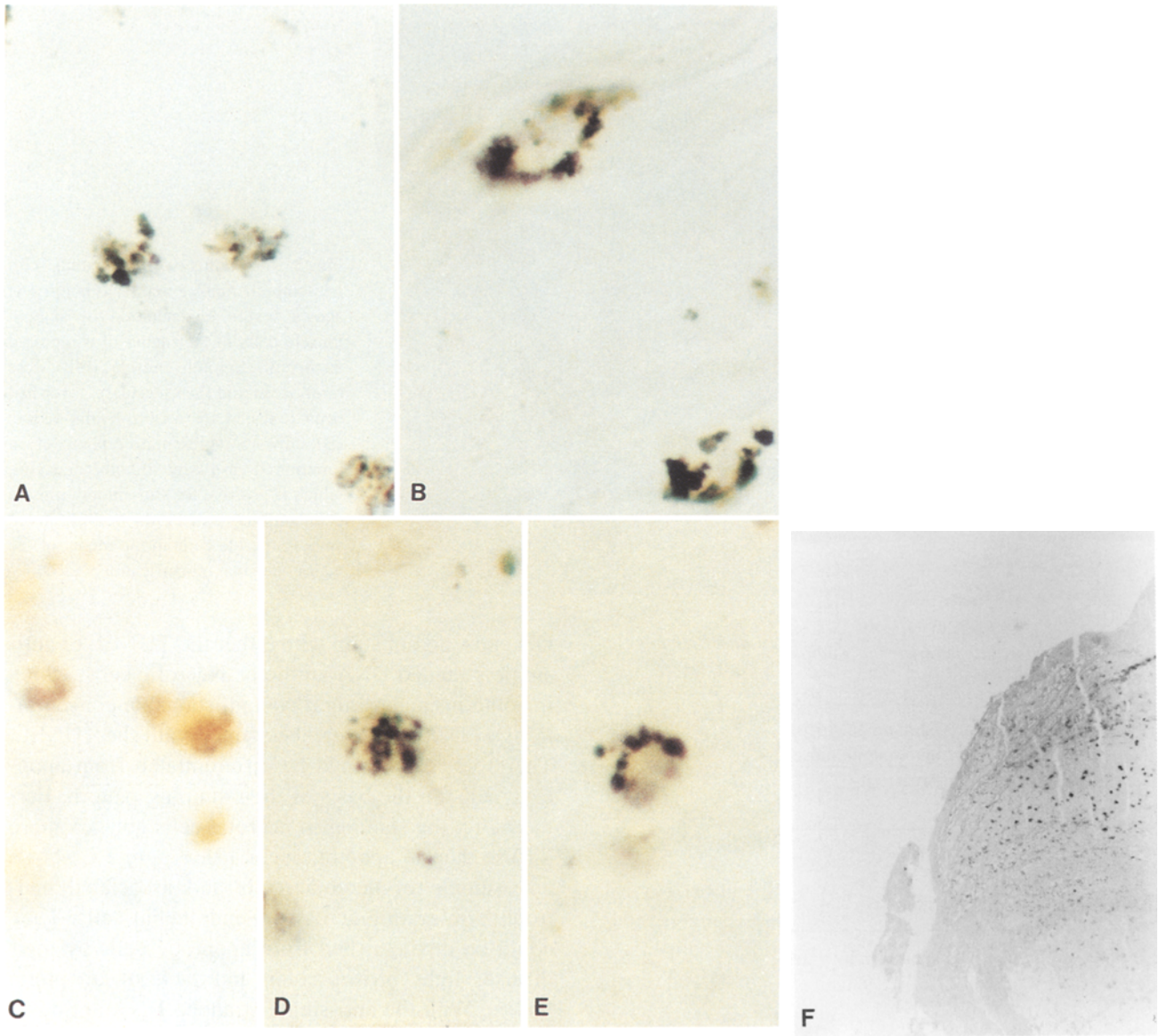

Fig. 3. DNA fragmentation with TUNEL (A–C) and with anti-single-stranded DNA (D, E) in smooth muscle cells in the media of a ruptured aneurysm. The light microscopic identification of apoptosis depended upon the recognition of round or oval bodies (apoptotic bodies) (A, D). These cells were observed in the lamina muscularis of the aneurysm (F: low-power microphotogram). The earliest recognizable stage in apoptotic formation involved chromatin condensation around the margin of the nucleus forming either crescent caps (B, E) or rings. Necrotic cells show a diffuse, light brown color, without apoptotic bodies, and associated neutrophils can be seen (C)

situ DNA 3′-OH labelling immunostaining (TUNEL) and anti-single-stranded DNA immunostaining are shown in Table 2. The same strict criteria as described above were used for these examinations. In the ruptured aneurysm wall, positive staining was observed in 54% (7/13) of the cases. DNA fragmentation, on the other hand, could generally not be detected in unruptured aneurysms with either examination. Both tests identified negative staining in 93% (13/14) of the unruptured aneurysms. Thus, DNA fragmentation was detected in situ in about half of the ruptured cases but rarely in unruptured cases.

Discussion

Immunohistochemical studies have shown that smooth muscle cells in the wall of ruptured aneurysms are subject to much more degeneration than those of unruptured aneurysms [14]. Our study showed, furthermore, that unruptured aneurysms with an angiographically identified smooth wall suffer less

degeneration than those with irregular shapes. Degeneration of the smooth muscle cells is likely to impair the strength of the wall and haemodynamic stress on the fragile wall can then produce an irregular shape. Thus, degeneration of the aneurysmal wall makes the wall frail, the shape irregular and increases the risk of rupture. We reported previously that unruptured aneurysms found together with ruptured aneurysms are more likely to rupture than those discovered incidentally, because smooth muscle cells in the dome of the former type are more degenerated than those of the latter [14]. These pathological findings indicate that an irregular shape and co-occurrence of a ruptured aneurysm should be considered risk factors for unruptured aneurysms.

This study found the first evidence of DNA fragmentation in the aneurysmal wall. The labelling target of TUNEL is the new 3′-OH DNA ends generated by DNA fragmentation, which are typically localized in morphologically identifiable nuclei and apoptotic bodies. Cells containing apoptotic bodies are referred as "apoptotic cells" [2, 17, 18, 20].

One should consider that necrotic cells may also contain stainable concentrations of DNA strand breaks, since DNA in the necrotic cells is degraded by lysosomal DNase. Positive signals identified with TUNEL may thus not be specific for apoptosis, as the kit can also stain such degraded DNA in necrotic cells. However, in the latter case the staining is more diffuse and apoptotic bodies are rarely present in necrotic cells, so that it is not too difficult to differentiate apoptotic cells from necrotic cells as reported by Li *et al.* [9].

To eliminate any doubts, however, other methods to detect DNA fragmentation should be included. One common method for identifying DNA fragmentation is agarose-gel-electrophoresis of DNA extracted from tissue showing ladder formation. We did not apply this method in this study, however, because the clinical specimens of the aneurysms were too small to collect any reliable samples. Therefore, we further assessed evidence of DNA fragmentation by using an anti-single-stranded DNA antibody [4, 6, 10, 11, 19]. This antibody recognizes 5 or 6 continuous nucleotides of single-stranded DNA and but not nick formation of double stranded DNA. Thus, degraded DNA of the necrotic cells will not be recognized. Tomei speculated that apoptosis involved modification of chromatin, resulting in a breakdown of the supercoiling organization and formation of individual superbeads [15]. He further proposed that single-stranded DNA in the nucleosomal linker region might constitute a critical early step in apoptosis. In other words, degradation of the supercoiling organization of DNA into single-strand formations might be a crucial step in apoptosis. Careful evaluation of the results with TUNEL and anti-single stranded DNA antibody showed good correlation and indicated the presence of apoptotic bodies in the aneurysmal wall.

We found that ruptured aneurysmal walls exhibit not only degeneration of the smooth muscle cells but also a well-defined evidence of DNA fragmentation suggesting apoptosis. Furthermore, these cells localize mainly to the ruptured domes of the aneurysms. We detected DNA fragmentation in situ in 54% of the ruptured aneurysms with both the TUNEL method and the anti-single stranded antibody, while only 7% of the unruptured ones showed positivity with both methods. Interestingly, the only unruptured aneurysm with positive results was accompanied by a ruptured aneurysm. This result suggests that apoptosis might be closely associated with rupture of the aneurysm.

The exact events initiating DNA fragmentation in the aneurysmal wall have not yet been identified. Apoptotic bodies have been detected after ischaemia in brain [9], liver [5], kidney [13], and in smooth muscle cells [1, 7, 8]. The dome of aneurysm is, of course, subjected to chronic ischaemic damage due to hypertension or atherosclerosis, which in turn may be the result of mechanical stress caused by jet flow and turbulence in the aneurysm. We found apoptotic bodies mainly in the ruptured cases but not in all of them. DNA fragmentation could be detected 18 hours after focal ischaemia in animal experiments [16]. The apoptotic bodies became visible 1–2 days after the initiating event and were found to persist for 16–20 days in those studies [5, 9, 13]. Therefore, apoptotic bodies might not be detected if the initiating events are intermittent and studies are performed subsequently, since only recent changes can be detected as apoptotic bodies. Repeated cell death in the wall would lead to final degeneration, while an ongoing dynamic process of apoptotic cell death might be involved in rupture of the aneurysm. It might be argued that the expression of apoptotic bodies is induced as a result of subarachnoid haemorrhage (SAH) as we found apoptotic bodies mainly in the ruptured cases. If the DNA fragmentation detected in this study had been caused by SAH, almost all ruptured cases should have been positive as the aneurysmal domed were resected 1 to 3 days after the onset of SAH. However, we cannot entirely exclude this pos-

sibility, so that a more extensive study of more cases is necessary.

Aneurysms, in many cases, have been observed to increase markedly in size before rupture [3]. Juvela *et al.* reported that all aneurysms that ruptured later increased in size, while aneurysms that did not rupture changed little in size during the follow-up period. However, they could not find any association between growth rate and blood pressure, patient age, size of aneurysm, or angiographic follow-up time [3]. An active ongoing process might well be involved in aneurysmal growth and rupture. In this connection, we showed a close relation between the irregular shape of aneurysms and loss of smooth muscle actin. Although we did not directly observe aneurysmal growth in our series, we speculate that apoptosis and degeneration of the smooth muscle cells can cause fragility of the wall, leading to growth or rupture, and apoptosis might thus be closely related to further destruction of the wall. Further studies are necessary to elucidate this aspect of aneurysm rupture.

Acknowledgements

We would like to thank Drs. T. Sugiyama, N. Miura, and Y. Kawarada, Department of Biochemistry, School of Medicine, Akita University, for donating anti-single-stranded DNA antibody. Appreciation is also extended to Drs. Mamoru Taneda and Mamoru Ito for providing pathological materials, and to Chiyako Imai for executing the immunohistochemical procedures.

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Comments

Sakaki *et al.* present interesting data on the regressive changes in the walls of ruptured and unruptured aneurysms. Loss of smooth muscle cells and DNA fragmentation labelled by immunohistochemistry were used as indicators for tissue degeneration. These phenomena were observed predominantly in ruptured aneurysms and in the unruptured aneurysms featuring an irregular shape. It is suggested that these regressive phenomena at a cellular level are responsible for growth and rupture of aneurysms and that identification of these phenomena in unruptured aneurysms could eventually be used to define which aneurysms will proceed towards rupture and which ones will not. I am more sceptical about such a clear causal relationship. I think in terms of different stages of evolution, through which enery aneurysm will pass sooner or later. During the first, the shape remains regular and the wall remains quite homogeneous. During the second stage, the wall with an increasing diameter develops relatively weak spots due to the increasing tension,

which are compensated for by the formation of daughter aneurysms and the apposition of thrombotic and scar tissue. I see the regressive changes of the normal vessel wall elements more as a phenomenon accompanying these reparative mechanisms [1].

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H. J. Steiger

In their study the authors compare the angiographic, histological and immunohistochemical findings of 13 ruptured and 14 unruptured intracranial aneurysms. The authors report, that unruptured aneurysms contained a muscularis layer, whereas ruptured aneurysms did not and that apoptotic bodies in the vessel wall were found in significantly more cases of ruptured aneurysms as compared with unruptured aneurysms. The group of unruptured aneurysms was devided into aneurysms with an irregular shape and those with a smooth wall. Irregularily shaped aneurysms were found to contain significantly fewer smooth muscle cells that those with a smooth wall. From these findings the authors speculate, that apoptosis of smooth muscle cells in the muscularis layer may lead to growth and rupture of the aneurysms.

Questions to the authors:

Firstly, if apoptosis of smooth muscle cells would lead to growth and rupture of the aneurysms, apoptotic bodies should be present in irregularily shaped aneurysms which were found to contain fewer muscle cells, but this was not demonstrated.

Secondly, apoptotic bodies should be present at the time of the rupture of the aneurysm, but the authors report that the apoptotic bodies became visible not before 1–2 days after the rupture of the aneurysm.

The the authors point out themselves that the apoptotic bodies might rather be the consequence than the cause for the rupture of the aneurysm. Accordingly there is no need to assume an independent degeneration process for the smooth muscle cells in the lamina mucularis of aneurysms; the decreased number of smooth muscle cells in irregularily shaped and ruptured aneurysms may be explained as the result of the haemodynamic stress and the extravasation of blood plasma into the wall of the artery leading to an impaired oxygen supply of the cells and consequent degeneration.

D. Stavrou, C. Hagel

Answers:

To the first question:

Indeed, we could not find apoptotic bodies in most of the unruptured aneurysms included in this study. Alteration of aneurysmal shape should be the result of wall fragility. The process making the wall fragile should thus have already happened. Therefore we could find few muscle cells and no apoptotic bodies in those aneurysms.

To the second question:

We described in the discussion that our materials were obtained 1 to 3 days after the onset of SAH and also with references that the apoptotic bodies became visible 1–2 days after the initiating event and were found to persist for 16–20 days in those studies. The initiating event could be confused with the rupture of the aneurysm. In our opinion, apoptosis should have been triggered for some unidentified reasons and should be visible at the time of rupture if we could obtain samples.

To the final question:

As we discussed, we cannot entirely exclude the possibility that apoptotic cells are rather the consequence of SAH. Cellular necrosis caused by the reasons alluded to can of course happen. Indeed, we observed some necrotic cells in the wall. However, if necrosis is the main feature of wall degeneration, there should be more inflammatory reaction than existed. Furthermore, the wall might be later thickened as a consequence of fibrosis. Apoptosis is silent cell death without any inflammatory reaction. We propose a possible involvement of apoptosis in the rupture of aneurysms.

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