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A novel arterial pouch model of saccular aneurysm by concomitant elastase and collagenase digestion*

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Abstract: Background: An ideal aneurysm model of cerebral aneurysm is of great importance for studying the pathogenesis of the lesion and testing new techniques for diagnosis and treatment. Several models have been created in rabbits and are now widely used in experimental studies; however, every model has certain intrinsic limitations. Here we report the development of a novel saccular aneurysm model in rabbits using an arterial pouch that is subject to in vitro pre-digestion with combined elastase and collagenase. Methods: A segment of right common carotid artery (CCA) was dissected out and treated with elastase (60 U/ml, 20 min) followed by type I collagenase (1 mg/ml, 15 min) in vitro. The graft was anastomosed to an arterial arch built with the left CCA and the remaining right CCA, while the other end of the graft was ligated. The dimension and tissue structure of the pouch were analysed immediately, 2 or 8 weeks after operation. Findings: Ten terminal aneurysms were produced. The gross morphology of the aneurysm resembles the human cerebral terminal aneurysms. We have observed the following pathological changes: (1) growth of the aneurysm (mean diameter increased from (2.0±0.1) to (3.2±0.3) mm at 2 weeks, $P<0.001$, $n=7\sim10$); (2) thinning of the aneurysmal wall (the mean wall thickness decreased to 44% at 2 weeks), which was accompanied by significant losses of elastic fibres, collagen and the cellular component; and (3) spontaneous rupture (3 out of 9, one aneurysm ruptured 24 h after operation with the other two at 2 and 4 weeks respectively). Conclusion: This rabbit arterial pouch model mimics human cerebral aneurysms in relation to morphology and histology. In particular, this model exhibited an increased tendency of spontaneous rupture.

Key words: Animal model, Collagenase, Elastase, Intracranial aneurysm
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INTRODUCTION

Intracranial aneurysms occur in approximately 1% of the population and are the most common cause of non-traumatic subarachnoid haemorrhage, which is a life-threatening emergency with high morbidity and mortality (Weir, 2002). Both genetic and environmental factors have been proposed to contribute to the development of the disease, whereas the cellular and molecular mechanisms of the pathogenesis and evolution (i.e. the initiation, enlargement and rupture) of aneurysms are largely unclear (Krex *et al*., 2001). Advancement in our understanding of the underlying mechanisms depends on suitable animal models that can reliably reflect the biological behaviour of human aneurysms. Moreover, models that precisely mimic the morphology, histology and haemodynamics of human aneurysms are also vital for the development of new endovascular techniques for the treatment of this disease.

Currently, two types of experimental aneurysm are commonly used in research. One is the venous pouch model as described by Strother *et al*.(1992). This model is constructed by anastomosing a segment of excised vein graft onto an endogenous artery. Advantages of this model include that the shape of the

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aneurysmal body resembles human saccular aneurysms, and it allows to manipulate the ratio of size of the aneurysm to its parent artery easily by selecting grafts with different dimensions. Although this model has been used to study the haemodynamics (Hashimoto, 1993) and cellular and molecular mechanisms (Hong *et al*., 2001; Raymond *et al*., 2005) of the aneurysm, an obvious intrinsic defect of this model is that the biological properties of arterial and venous cells are significantly different. Moreover, the intact elastic lamina and tunica media render this model to be histologically different from the human disease (Abruzzo *et al*., 1998).

The other aneurysm model was developed with intra-luminal perfusion with pancreatic elastase in arteries (Cawley *et al*., 1996; Cloft *et al*., 1999), and this method has subsequently been modified in different ways by other researchers (Hoh *et al*., 2004; Krings *et al*., 2003). The aneurysms created with these methods have been shown to have similar geometric (Short *et al*., 2001) and histological (Abruzzo *et al*., 1998) features to human intracranial aneurysms. Particularly, destruction of the internal elastic laminar can be readily achieved after elastase digestion (Abruzzo *et al*., 1998; Cawley *et al*., 1996; Krings *et al*., 2003). This model has been used in evaluating novel endovascular treatment techniques (Dai *et al*., 2005; Hans *et al*., 2003; Kallmes *et al*., 2003). Despite these, however, spontaneous rupture of the aneurysm is infrequent; thus the natural history of human intracranial aneurysm is not precisely reproduced by this model.

In the present study, we explored to create an arterial pouch model of saccular aneurysm in rabbit carotid arteries by pre-digesting the artery graft with combined elastase and collagenase in vitro. Aneurysms created with this method are similar to human aneurysms in morphology and histology. Particularly, this model has an increased tendency of spontaneous rupture as compared to previous ones.

MATERIALS AND METHODS

Animals

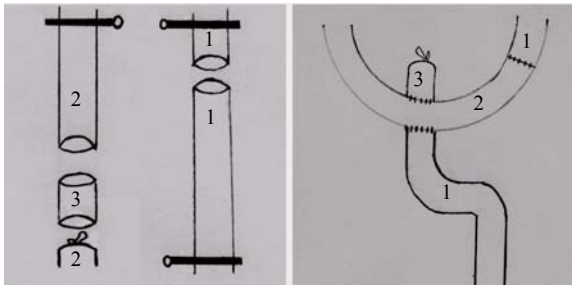
Experimental aneurysms were created in ten New Zealand White rabbits (body weight 2.7~3.3 kg). The protocol was approved by local Animal Ethics Committee. Animals were pre-medicated with intramuscular ketamine (5 mg/kg) and atropine (0.04 mg/kg). General anaesthesia was achieved by intravenous injection of pentobarbital sodium (25 mg/kg). Prophylactic endotracheal intubation was also performed to cope with any loss of spontaneous breathing. The blood pressure and heart rate were monitored continuously during the operation. All of the surgical procedures were performed under sterile conditions.

Aneurysm creation

An incision was made in the middle of the neck and both left and right common carotid arteries (CCAs) were exposed. A segment of approximately 8 cm long was cleared in each artery. The proximal end of the right CCA was ligated with a 3-0 suture while the distal end was temporarily clamped. An arterial segment of 8 mm long was harvested from the right CCA immediately distal to the ligation site (Fig.1). The isolated segment was incubated in a Hanks solution containing elastase (60 U/ml, Sigma, USA) for 20 min followed by type I collagenase (1 mg/ml, Sigma, USA) for 15 min. Meanwhile, the left CCA was clamped at both proximal and distal ends and cut at the distal quarter of the length (Fig.1). The free end of the distal part of the right CCA was anastomosed to that of the left CCA such that a U-shaped arterial arch was created. Two arteriotomies were made at the bottom of the arch on both caudal and rostral sides respectively, and the free end of the proximal part of left CCA was then anastomosed to the caudal orifice in the arch. The aneurysm was created by suturing the elastase/collagenase-treated artery graft onto the rostral orifice, while the other end of the graft was ligated permanently (Fig.1). Because the artery graft was very floppy and could not stay in shape after enzymatic digestion, the anastomosis of this was difficult. Therefore, the graft was mounted onto a plastic tubing, which served as a scaffold during anastomosis and removed after the procedure. Penicillin was administered for 3 d after operation to prevent infection.

Pathological examination

The diameter of the aneurysm was measured immediately after construction and at the time of sample harvesting. One animal was sacrificed immediately after the operation; the other nine animals



(a) (b)

Fig.1 Diagram illustrating the procedure of construction of the arterial pouch aneurysm. (a) Disposal of bilateral carotid arteries; (b) Formation of terminal aneurysm

1: Left common carotid artery (CCA); 2: Right CCA; 3: The 8-mm long arterial segment obtained from right CCA to be used as the pouch. The upper side of the diagram represents head, whereas the bottom represents heart. The hairpins indicate clamps

were allowed to recover and sacrificed at 2 or 8 weeks after operation. For histological examination, the aneurysms together with the parent arteries were dissected out, fixed in 2% paraformaldehyde for at least 24 h, and paraffin sections of 5 μm thickness were cut. The general tissue structure was examined with haematoxylin-eosin (HE) staining; Victoria Blue and Masson staining were used to demonstrate elastic laminar and collagen respectively.

Statistical analysis

Data are presented as mean±standard deviation (*SD*). The mean data between groups were analysed with unpaired *t*-test; $P<0.05$ was considered to be statistically significant.

RESULTS

A total of 10 terminal aneurysms were successfully created using the method as described, with the length of operation being about 3 h. The animal mortality during the procedure was 0%.

Growth of the aneurysms

Obvious enlargement of the aneurysmal sac was observed at 2 weeks in all of the aneurysms examined (Figs.2a and 2b). The mean diameter of the aneurysms was (2.0±0.1) mm (n=10) immediately after construction and (3.2±0.3) mm (n=7) at 2 weeks respectively ($P<0.001$).

Spontaneous rupture

Three spontaneous ruptures out of a total of 9 aneurysms were observed within the 8-week period of follow up. One aneurysm ruptured 24 h after construction; the second one ruptured at 2 weeks and the third one at 4 weeks after construction. The gross appearance of a ruptured aneurysm (at 2 weeks) is shown in Fig.2c.

Histology of the aneurysm

HE staining of transverse sections of the arterial wall immediately after enzymatic digestion revealed that the thickness of the wall was comparable with that of normal arteries. Most of the cellular component was well preserved after digestion (Fig.2d). In contrast to normal arteries, the organisation of extracellular matrices was deranged and thus the connections among cells looked loose and the orientations of different vascular cells became irregular. The typical differentiation of tunica intima, media and adventitia in HE-stained sections was lost. Fragmentation of elastic laminar could be observed. There was a progressive thinning of the aneurysmal wall (Fig.2e). The average thickness of the aneurysmal wall at 2 weeks was (79±7) μm, which was 44% of that of normal carotid arteries [(178±8) μm] ($P<0.001$, unpaired *t*-test, n=7~10). Most part of the aneurysmal wall at 8 weeks was only composed of a thin layer of acellular fibrous tissue (Fig.2f). Partial thrombi were found in 3 (out of a total of 9) aneurysms at the conclusion of the experiment. A remarkable alteration of the aneurysmal wall was the dramatic losses of the elastic laminar and collagen content, as revealed by Victoria Blue and Masson staining (Fig.3).

DISCUSSION

Aneurysm models built in smaller laboratory animals such as rabbits, as compared to swine or canine models (Dai *et al*., 2005; Shin *et al*., 2005), are associated with advantages such as free access and low cost for maintenance. More importantly, it has been recognised that the coagulation system of rabbits is similar to that of humans, and the size of rabbit carotid artery is comparable to that of the human middle cerebral arteries (Abruzzo *et al*., 1998). In this study, we have created a novel saccular aneurysm

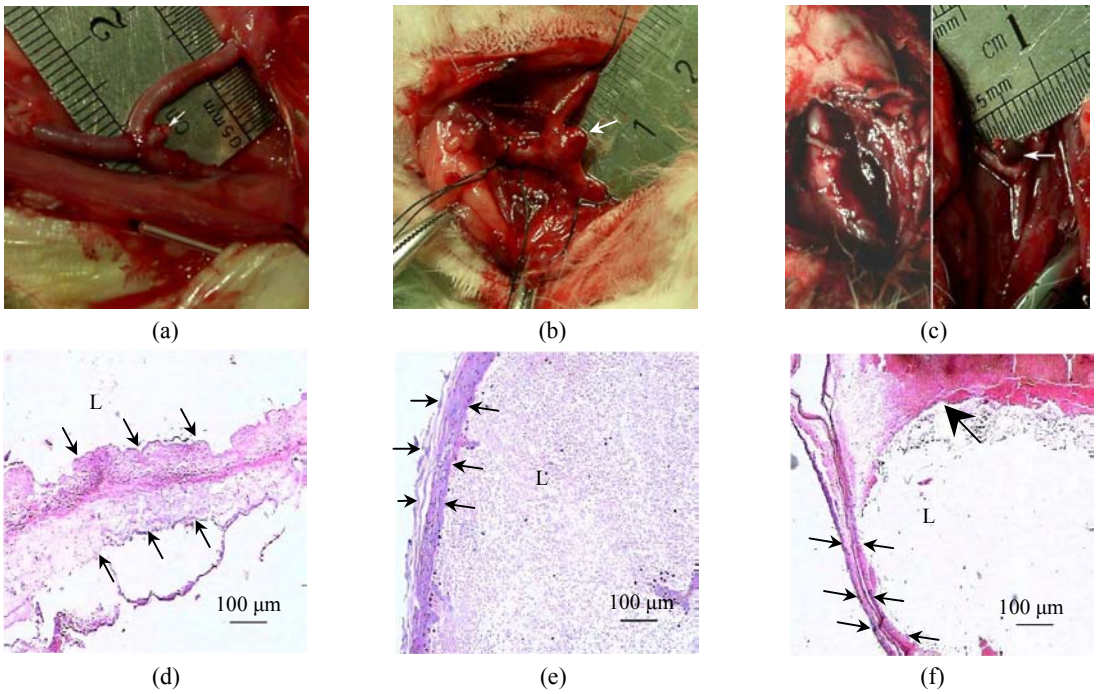


Fig.2 Gross appearance (a, b and c) and histology (d, e and f) of the arterial pouch aneurysms. (a) An arterial pouch aneurysm (white arrow) immediately after creation; (b) The same aneurysm examined 2 weeks after creation. There was an obvious enlargement of the sac (white arrow); (c) An aneurysm with spontaneous rupture. The animal died at 2 weeks. Post-mortem examination revealed a ruptured aneurysm. The left panel shows that the aneurysm was buried under the haematoma; the right panel shows the aneurysmal body (white arrow) after clearance of the blood clot. (d, e and f) HE-stained tissue sections of the aneurysmal wall (defined by small black arrows) collected immediately (d), 2 weeks (e) and 8 weeks (f) after creation. There was a gradual thinning and decrease in cellularity of the wall. It is noted that at 8 weeks, most part of the aneurysmal wall was only composed of a thin layer of acellular fibrous tissue. A thrombus was found in the sac (large arrow in 2f). L indicates the lumen

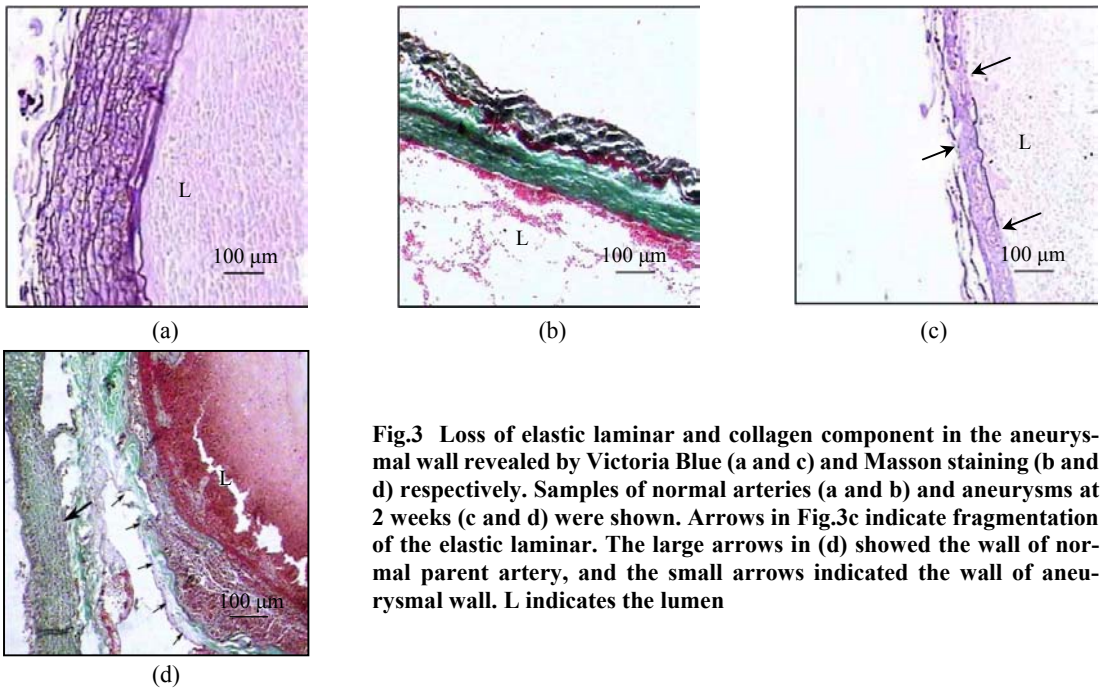


Fig.3 Loss of elastic laminar and collagen component in the aneurysmal wall revealed by Victoria Blue (a and c) and Masson staining (b and d) respectively. Samples of normal arteries (a and b) and aneurysms at 2 weeks (c and d) were shown. Arrows in Fig.3c indicate fragmentation of the elastic laminar. The large arrows in (d) showed the wall of normal parent artery, and the small arrows indicated the wall of aneurysmal wall. L indicates the lumen

model in rabbit carotid arteries by using an arterial pouch that is pre-digested in vitro with combined elastase and collagenase. This model exhibits a high similarity in morphology and histology to the naturally occurring human aneurysms. Another advantage of our model is that the cerebral blood supply from both CCAs is preserved via the artificial arterial arch, which may help to prevent possible neurological complications caused by carotid artery ligation (Hoh *et al*., 2004).

The venous pouch model created in rabbit carotid arteries has been used to simulate the morphology of human intracranial saccular aneurysms and may be useful in haemodynamic studies (Hashimoto, 1993). However, a number of studies have clearly demonstrated that the histology of this model is largely different from that of human aneurysms, including the intact internal elastic lamina and tunica media, extensive inflammatory response and prominent neointimal thickening (Abruzzo *et al*., 1998; Stehbens, 1981). To mimic the disruption of internal elastic lamina, a hallmark of human intracranial aneurysms, intra-luminal infusion with elastase has been used in various experimental models to create saccular aneurysms (Altes *et al*., 2000; Dai *et al*., 2005; Kallmes *et al*., 2002; Short *et al*., 2001). In addition to the disrupted internal elastic lamina, histological studies have revealed that other features of human cerebral aneurysms are also well preserved in this model, including thinning of the wall, medial atrophy, and loss of cellular elements, with minor inflammation and intimal proliferation (Abruzzo *et al*., 1998; Hoh *et al*., 2004). In the present study, histological examination demonstrated that all of these characteristic features of elastase-induced aneurysms were readily observed in our arterial pouch model. Moreover, inflammation and neointimal proliferation were not observed in our model.

Progressive growth of the aneurysmal sac is a characteristic biological behaviour of human cerebral aneurysms. The size of the aneurysm is an independent predictor of the risk of rupture (Juvela, 2002). We found that 100% of aneurysms created in our study enlarged in 2 weeks, with a mean diameter being 1.5-fold larger than that of immediately after creation. This spontaneous growth over time is consistent with previous observations in models based on in vivo intra-luminal elastase incubation, and the dimension of the final aneurysmal sac in our study is comparable to those reported by others (Ding *et al*., 2006; Fujiwara *et al*., 2001; Kallmes *et al*., 2002; Short *et al*., 2001). Although people have identified a number of risk factors that may predict intracranial aneurysm growth, the mechanism(s) underlying this process is largely unknown. Several studies have proposed that factors including structural changes of the arterial wall (Merei and Gallyas, 1980), blood pressure (Steiger *et al*., 1989), increased activity of matrix metalloproteinases (MMPs) (Papalambros *et al*., 2003; Petrinec *et al*., 1996) and increased haemodynamic stresses (Hoi *et al*., 2004), may all contribute to this behaviour of aneurysms. In our study, the aneurysmal growth of the artery was associated with a progressive degeneration of the arterial wall; together with the findings that human aneurysms are universally defective in the arterial wall structure, our data support the argument that attenuated mechanical strength of the aneurysmal wall is an essential element that predisposes them to expansion and rupture (Abruzzo *et al*., 1998).

Within a period of 8 weeks, 3 spontaneous ruptures out of a total of 9 aneurysms were observed and monitored continuously. The incidence of rupture in the present model is different from those observable in other models of elastase-induced aneurysms, in which spontaneous rupture of the aneurysms was rare (Abruzzo *et al*., 1998; Cawley *et al*., 1996; Cloft *et al*., 1999; Hoh *et al*., 2004; Kallmes *et al*., 1999; 2002; Krings *et al*., 2003; Short *et al*., 2001). Miskolczi *et al*.(1998) reported a novel rabbit aneurysm model by elastase digestion of the adventitia of intact arteries, in which spontaneous rupture was observed. However, the rupture only occurred in one aneurysm (among the 6 created) at 55 min after elastase treatment. We propose that the relative resistance to rupture of elastase-induced aneurysm in rabbit arteries may be due to the residual collagen in the vessel wall, which is a major structural component of blood vessels and important to maintain the tensile strength of the wall. There is evidence that alterations in either the quantity (Gaetani *et al*., 1997; 1998; Ostergaard and Oxlund, 1987; van den Berg *et al*., 2001) or quality (Whittaker *et al*., 1988) of collagen may be related to the development of cerebral aneurysms. In some patients with congenital cerebral aneurysms, the etiology of the disease has been ascribed to an inherited type III collagen deficiency (Ehlers-Danlos syndrome), which causes arterial fragility (Pope *et al*., 1981). Moreover,

a recent study has identified a significant association of familial intracranial aneurysm with a single nucleotide polymorphism (SNP) in the type I collagen gene (COL1A2), which results in an alanine to proline substitution at the amino acid 459 located on a triple-helical domain (Yoneyama *et al*., 2004). All these results indicate that in addition to elastin, changes in collagen may also be involved in the growth and rupture of cerebral aneurysms. To our knowledge, our study is the first to explore the use of combined elastase and collagenase to create experimental aneurysms. Although Miskolczi *et al*.(1998) reported that adventitial application of collagenase alone in rabbit arteries could not induce aneurysm formation, our study clearly shows that addition of collagenase in the step of enzymatic digestion of the rabbit artery makes the aneurysmal wall more prone to rupture, as compared to the elastase alone-induced models.

In this study, we adopted the method of treating the isolated arteries with enzymes in vitro. This procedure allows the researchers to precisely control the digestion conditions such as enzyme concentration, temperature and incubation time. Moreover, in contrast to the commonly used intra-luminal incubation, our method allows both of the tunica intima and adventitia to be in contact with the digestion solution. The results demonstrate that there is no obvious difference of the histological features of the arterial wall between the aneurysms created by in vitro and in situ digestion. Kallmes *et al*.(2002) determined the effects of varying elastase concentrations on the development of aneurysms in the ligated right CCAs in rabbits using intra-luminal incubation, and found that the elastase concentration and incubation time did not affect the size of resultant aneurysms. However, digestion with higher concentrations of elastase could induce unwanted dilatation of the adjacent parent arteries. This complication can be easily avoided by incubating the arterial graft in vitro. In addition, in vitro digestion eliminates the involvement of balloon catheters, as used by some investigators in order to prevent the adverse effects of elastase to the animals (Krings *et al*., 2003), making this model available to researchers without access to interventional technologies.

In summary, we have developed a new rabbit model of saccular aneurysm using elastase plus collagenase-digested arterial pouch, which is highly reproducible and technically less demanding. This model mimics human intracranial aneurysms in respect to morphology and histology. More importantly, this aneurysmal model has an increased tendency of spontaneous rupture. We suggest that this model may be useful in preclinical studies targeting aneurysms with a high risk of rupture.

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