Fusiform Aneurysm Model in Rabbit Carotid Artery

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Key Words

Rabbit • Carotid • Aneurysm • Matrix metalloproteinase-9 • Elastase

Abstract

Aims: To develop a reproducible and accessible model of elastase-induced fusiform aneurysm in carotid rabbit arteries. **Methods:** Elastase, at a concentration of 1–30 U, was incubated into the lumen of carotid rabbit arteries. Four weeks later, angiography, histomorphometry, immunohistochemistry and zymography were performed. **Results:** The optimal concentration of elastase in this model was 3 U according to the balance between mortality and thrombosis rates. Indeed, at 3 U, external carotid diameter increased from 1.9 ± 0.1 to 3.1 ± 0.4 mm ($p < 0.0001$) associated with degradation of elastic fibers, matrix metalloproteinase-9 secretion, apoptosis and macrophage infiltration. **Conclusions:** Our study underlines that abdominal aortic aneurysm can be reliably duplicated in an elastase-induced aneurysm in carotid artery, a much more accessible vessel.

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Introduction

Arterial aneurysms are characterized by an increase in external aortic diameter associated with fragmentation and degradation of elastic and collagen fibers, apoptosis of smooth muscle cells and infiltration of inflammatory cells. The most important mechanism involved in this destructive process is related to the overproduction of matrix metalloproteinases (MMP) [1–3].

Various experimental aneurysm models have been previously proposed, including venous graft, aortic xenograft, CaCl_2 periarterial application and endoluminal injection of elastase [4–13]. However, surgical procedure is often complex and the small size of the animals renders the access for local endovascular therapy difficult. Elastase-induced aneurysm has been used most frequently [8–13]. Elastase induces fragmentation and degradation of elastin network and arterial aneurysm formation [8–13]. Rabbit carotids or rat abdominal aortas were most frequently used to create this aneurysmal model. However, the distal permanent ligation of carotid, used in most models, leads to saccular aneurysms mimicking intracranial aneurysms thus adapted to neurosurgical experimentations [9–12]. In contrast, endoluminal local application of elastase in rat abdominal aorta induces fusiform aneurysms [8]. However, this location is not easily accessible for local endovascular therapy. We therefore

aimed to adapt the elastase-induced model without permanent ligation to develop a fusiform aneurysm model in rabbit carotid arteries.

Materials and Methods

Aneurysm Model Creation Procedure

The investigations conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

New Zealand white rabbits of 3–4 kg ($n = 42$) were anesthetized with xylazine (5 mg/kg) and ketamine (35 mg/kg) by intramuscular injection. A segment (1–2 cm) of the right common carotid artery ($n = 34$) was isolated using 2 transient ligatures. Six hundred microliters of porcine pancreatic elastase type I (Sigma-Aldrich, Saint-Quentin, France) were injected into the lumen of the carotid arteries during 15 min via a 24-G catheter. Four different concentrations of elastase (1, 3, 10 and 30 U/artery) were used in order to define the optimal concentration in our condition of experiments. Thereafter, arterial segments were washed twice with saline solution. The catheter was then removed, and closure of the puncture of the artery induced by the catheter was performed by a thread suture in order to re-expose the circulating blood flow. An X-ray opaque marker was placed at the surgical site. The left contralateral carotid arteries were used as control.

Arteriography

Four weeks after elastase incubation, a selective angiogram was performed by manual injection of 3–5 ml angiographic contrast agent via a 5-french angiographic catheter, as previously described [14].

Histological Analysis

After angiography, external carotid arterial diameters were measured by an electronic caliper. Animals were then sacrificed by intracardiac injection of an overdose of sodium pentobarbital under general anesthesia (120 mg/kg). Arteries were removed after in vivo fixation with 10% phosphate-buffered formaldehyde solution perfused at 100 mm Hg for 15 min. Each carotid artery was cut in consecutive slices of 2 mm from the proximal to the distal end, and embedded in paraffin. Transverse sections (5 μ m thick) were stained with orcein to visualize elastic network and perform morphometric analysis. Cross-sections of total vessel area, lumen area and the area circumscribed by the external elastic lamina were measured in elastase-treated and control segments (IPS 4.02 software; Tribvn, Chatillon, France), as previously described [14].

Elastin density was quantified in the media of the elastase-treated and control segments, as previously described (IPS 4.02 software, Tribvn, France) [14].

Immunohistochemistry

Identification of smooth muscle cells and macrophages, and evaluation of MMP-9 secretion were performed using the following specific primary antibodies: monoclonal mouse anti-human smooth muscle actin (Dako, Trappes, France); monoclonal mouse anti-rabbit RAM11 antibody (DakoCytomation, Carpinteria, Calif., USA); goat anti-mouse MMP-9 antibody (R&D Systems, Min-

Table 1. Incidence of lethality, thrombosis, or microscopic and macroscopic alterations in elastase-treated rabbit carotid arteries

	Lethality	Thrombosis	Microscopic alterations	Macroscopic alterations
Elastase 1 U ($n = 9$)	0 (0)	0 (0)	9 (100)	3 (33.3)
Elastase 3 U ($n = 12$)	3 (25)	3 (25)	12 (100)	6 (50)
Elastase 10 U ($n = 6$)	0 (0)	5 (83.3)	6 (100)	1 (16.7)
Elastase 30 U ($n = 7$)	3 (42.8)	3 (42.8)	4 (57.1)	3 (42.8)

Microscopic alterations were defined by elastin fragmentation or destruction. Macroscopic alterations were defined by increase in external arterial diameter. Figures in parentheses are percentages.

nesota, Minn., USA). Sections not treated with primary antibody were used as negative control. Slices were then treated with a horseradish peroxidase-conjugated anti-mouse or anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), dilution 1/500, incubated for 30 min, revealed then with diaminobenzidine solution (DakoCytomation).

In situ detection of apoptotic cells was performed using a rabbit monoclonal antibody against human cleaved caspase-3 (Cell Signaling Technology, Danvers, Mass., USA), as previously described [15].

MMP-2, MMP-9 and TIMP-1 Analysis

In these experiments, carotid arteries were retrieved without in vivo fixation. Arteries were kept in DMEM without serum for 24 h. Then, protein extract and conditioned media (supernatant) were collected for biochemical analysis. MMP-2, MMP-9 and TIMP-1 secretion were analyzed by zymography or Elisa (Quantikine; R&D Systems) in the supernatant and the carotid tissue, as previously described [16]. MMP-9/TIMP-1 complexes were also quantified using an enzyme-linked immunosorbent assay kit (DY1449; R&D Systems).

Statistical Analysis

Results were expressed as means $\pm$ SD. Lethality and thrombosis occurrence were evaluated by a Fisher exact test. Comparison among the different groups of arteries (control and elastase-treated arteries) was evaluated by an ANOVA test. If global significant difference was found, a PLSD Fisher test was used to identify differences between groups. Correlations between pairs of factors were evaluated using Spearman's rank correlation. Values were considered statistically different when $p < 0.05$.

Results

Evaluation of the Effect of Elastase Concentration

Mortality was significantly higher in arteries incubated with 30 U of elastase than those injected with other concentrations ($p = 0.006$; table 1). Furthermore, acute

Fig. 1. Angiographic demonstration of aneurysm creation. Arteriography of rabbit carotid artery 28 days after porcine pancreatic elastase incubation in the right carotid artery exhibited luminal enlargement (arrow) compared with control left carotid artery.

occluded thrombosis frequently occurred with 30 U and 10 U of elastase compared to smaller concentrations (that is, 1 and 3 U; 62 vs. 14%; $p = 0.002$). Moreover, no significant variation of carotid external diameter was observed with 1 U of elastase. We therefore believed that the optimal concentration of elastase was 3 U in our conditions of experiments, and we investigated more precisely the effect of this concentration in the arterial wall.

Arterial Measurements

External carotid diameter significantly increased in elastase-treated arteries (3 U) compared to control arteries (3.1 ± 0.4 vs. 1.9 ± 0.1 mm, $p < 0.0001$). Angiography confirmed fusiform lumen enlargement as shown in figure 1.

Histomorphometry analysis showed that total arterial and lumen areas, as well as the area circumscribed by external elastic lamina, were significantly increased in elastase-treated arteries compared to control arteries (fig. 2).

Finally, elastin fibers were fragmented and altered, and elastin density was significantly decreased compared to control arteries (fig. 3, 4). Elastic network was severely degraded with internal and external elastic laminae and medial rupture with high doses of elastase (data not

Fig. 2. Histomorphometry. Bar graph representation of the morphometric results in elastase-treated carotid arteries at 4 concentrations (1, 3, 10 and 30 U) compared with control (C) untreated arteries: total arterial area (a), luminal area (b) and area circumscribed by external elastic lamina (EEL; c). Results are expressed as means $\pm$ SD. * $p < 0.05$ vs. control, + $p < 0.05$ vs. 1 U; ° $p < 0.05$ vs. 10 U.

Fig. 3. Elastin network. Example of control (**a** and **b**) and treated carotid arteries with 1 U (**c** and **d**), 3 U (**e** and **f**), 10 U (**g** and **h**) and 30 U (**i** and **j**) of elastase. Arteries were stained with orcein. $\times 2.5$ (**a**, **c**, **e**, **g**, **i**); $\times 40$ (**b**, **d**, **f**, **h**, **j**).

shown). Interestingly, we found a significant negative correlation between elastin density and total arterial area ($r = -0.60$, $p = 0.03$).

Immunohistochemistry

Macrophage infiltration (RAM-11), apoptosis (cleaved caspase-3) and MMP-9 were detected in carotid elastase-treated arteries, but not in control arteries (fig. 5). Smooth muscle cells were present in the media of control and elastase-treated arteries (fig. 5a, b). Macrophages were predominantly detected in the neointima (fig. 5c, d), whereas extracellular MMP-9 immunostainings were detected in the media and adventitia (fig. 5e, f). Apoptosis was present in the neointima close to the lumen of the arteries (fig. 5g, h).

MMP-2, MMP-9 and TIMP-1 Analysis

As compared to control arteries, we observed a significant increase in MMP-9 and a significant decrease in TIMP-1 secretions in the supernatant as well as in the carotid tissues of elastase-treated carotid arteries (fig. 6a–c). Zymography showed that both pro and active forms of MMP-9 were increased (fig. 6a). In contrast, MMP-2 was similar in elastase-treated and control arteries (fig. 6a). Moreover, MMP-9/TIMP-1 complexes were significantly higher in elastase-treated carotid arteries than in control arteries (fig. 6d).

Discussion

We aimed to develop an accessible and reproducible model of elastase-induced fusiform aneurysm in carotid rabbit arteries in order to mimic abdominal aortic aneurysm, making it possible to evaluate local cell therapy in the future.

We first concentrate our work on the determination of the optimal concentration of porcine pancreatic elastase incubation in rabbit arteries. In carotid arteries, the lowest used concentration of elastase (1 U) was well tolerated but was unable to induce arterial enlargement. In contrast, the highest concentrations of elastase (10 U and more clearly 30 U) induced fusiform aneurysms but were associated with high mortality and thrombosis rates, as previously reported by other groups using high doses of elastase [17]. Finally, the optimal balance between mortality and thrombosis and aneurysm formation was obtained with the concentration of 3 U of porcine pancreatic elastase. We therefore investigated more specifically the impact of this concentration to the arterial wall.

Fig. 4. Elastin density. Bar graph representation of elastin density in elastase-treated and control (C) arteries. Results are expressed as means $\pm$ SD. * $p < 0.05$ vs. control; + $p < 0.05$ vs. 1 U.

At 3 U of elastase, a 50% increase in arterial diameter was obtained, and elastic fibers were clearly fragmented. Even 4 weeks after elastase incubation, an infiltration of macrophages was present, and apoptosis could be detected as previously reported in human abdominal aortic aneurysm [3]. Ex vivo analysis of the aneurysmal carotid revealed, as observed in the human disease, an increase in MMP-9 activity within the tissue and its secretion within the surrounding medium [3, 16].

Even if MMP-9/TIMP-1 complexes formation was significantly increased in elastase-treated carotid arteries both in the tissue and in the medium, this was not sufficient to compensate the lysis activity of MMP-9 on the elastic network. This may be due to the fact that TIMP-1 was increased at a tissue level but not sufficiently secreted to efficiently inhibit MMP-9, and to prevent extracellular matrix degradation [3, 16, 18]. We therefore concluded that by using 3 U of porcine pancreatic elastase incubation in rabbit carotid arteries, we get a well-tolerated and reproducible model mimicking the main characteristics of abdominal aortic aneurysm.

Limitations

Models using elastase to induce aneurysm do not show clear atherosclerotic changes, and this limitation needs to be taken into account in the transposition of the data to the ‘true human degenerative aneurysm’.

Fig. 5. Immunohistochemistry. Example of rabbit carotid artery incubated with porcine pancreatic elastase (3 U). **a, b** Smooth muscle cells (mouse monoclonal antibody against human smooth muscle actin; Dako). **c, d** Macrophages (monoclonal mouse antibody against rabbit RAM-11; DakoCytomation). **e, f** MMP-9 (monoclonal goat antibody against mouse MMP-9; R&D Systems). **g, h** Apoptosis (rabbit monoclonal antibody against human cleaved caspase-3; Cell Signaling Technology); **i, j** Sections not treated with primary antibody were used as negative control. $\times 5$ (**a, c, e, g, i**); $\times 20$ (**b, d, f, h, j**). Positive stainings are indicated by arrows (**e-g**). Counterstaining with hematoxylin (**a-d, i, j**).

Fig. 6. MMP-9 and TIMP-1 secretion. **a** Example of zymogram showing the increase in MMP-9 (active and pro forms) in supernatant and tissue of elastase-treated arteries (3 U). **b–d** Bar graph representation of MMP-9 (**b**), TIMP-1 (**c**) and MMP-9/TIMP-1 complexes (**d**) analyzed by ELISA in control and elastase-treated arteries (3 U). Results are expressed as means $\pm$ SD. * $p < 0.05$.

Moreover, we only evaluated one period of endoluminal incubation (that is, 15 min) of elastase in the present study in order to simplify the surgical protocol. However, it has been previously reported that the period of elastase incubation correlated with the severity of the destruction of the extracellular matrix [17, 19]. Finally, we did not use a true contralateral artery, since it was not temporarily occluded and injected with a saline solution. However, this control has already been evaluated by others [8]. In addition, in our

experimental conditions, we verified that incubation with a saline solution during 15 min in rabbit carotid artery did not alter elastin network, induce MMP-9 secretion, macrophage infiltration and apoptosis [unpubl. data].

In conclusion, the outcome of our study underlines that findings of elastase-induced abdominal aortic aneurysms were reliably duplicated in rabbit carotid arteries. This easy accessible and reproducible model may be helpful to evaluate the effect of new therapeutic strategies.

References

- 1 Pyo R, Lee JK, Shipley JM, Curci JA, Mao D, Ziporin SJ, Ennis TL, Shapiro SD, Senior RM, Thompson RW: Targeted gene disruption of matrix metalloproteinase 9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms. *J Clin Invest* 2000;105:1641–1649.
- 2 Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT: Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms. *J Clin Invest* 2002;110:625–632.
- 3 Sakalihasan N, Limet R, Defawe OD: Abdominal aortic aneurysm. *Lancet* 2005;365:1577–1589.
- 4 Guglielmi G, Ji C, Massoud TF, Kurata A, Lownie SP, Viñuela F, Robert J: Experimental saccular aneurysms. II. A new model in swine. *Neuroradiology* 1994;36:547–550.
- 5 Allaire E, Bruneval P, Mandet C, Becquemin JP, Michel JB: The immunogenicity of the extracellular matrix in arterial xenografts. *Surgery* 1997;122:73–81.
- 6 Allaire E, Hasenstab D, Kenagy RD, Starcher B, Clowes MM, Clowes AW: Prevention of aneurysm development and rupture by local overexpression of plasminogen activator inhibitor-1. *Circulation* 1998;98:249–255.
- 7 Gertz DS, Kurgan A, Eisenberg D: Aneurysm of the rabbit common carotid artery induced by periarterial application of calcium chloride in vivo. *J Clin Invest* 1988;81:649–656.
- 8 Anidjar S, Salzmänn JL, Gentric D, Lagneau P, Camilleri JP, Michel JB: Elastase-induced experimental aneurysms in rats. *Circulation* 1990;82:973–981.
- 9 Cawley CM, Dawson RC, Shengelaia G, Bonner G, Barrow DL, Colohan ART: Arterial saccular aneurysm model in the rabbit. *Am J Neuroradiol* 1996;17:1761–1766.
- 10 Krings T, Möller-Hartmann W, Hans FG, Thiex R, Brunn A, Scherer K, Meetz A, Dreeskamp H, Stein KP, Gilsbach JM, Thron A: A refined method for creating saccular aneurysm in the rabbit. *Neuroradiology* 2003;45:423–429.
- 11 Thiex R, Hans FJ, Krings T, Möller-Hartmann W, Brunn A, Scherer K, Gilsbach JM, Thron A: Haemorrhagic tracheal necrosis as a lethal complication of an aneurysm model in rabbits via endoluminal incubation with elastase. *Acta Neurochir* 2004;146:285–289.
- 12 Kallmes DF, Fujiwara NH, Berr SS, Helm GA, Cloft HJ: Elastase-induced saccular aneurysms in rabbits: a dose-escalation study. *Am J Neuroradiol* 2002;23:295–298.
- 13 Hoh BL, Rabinov JD, Pryor JC, Ogilvy CS: A modified technique for using elastase to create saccular aneurysms in animals that histologically and hemodynamically resemble aneurysm in human. *Acta Neurochir* 2004;146:705–711.
- 14 Lafont A, Durand E, Samuel JL, Besse B, Addad F, Levy BI, Desnos M, Guerot C, Boulanger CM: Endothelial dysfunction and collagen accumulation: two independent factors for restenosis and constricting remodeling after experimental angioplasty. *Circulation* 1999;100:1109–1115.
- 15 Durand E, Scoazec A, Lafont A, Boddaert J, Al Hajzen A, Addad F, Mirshahi M, Desnos M, Tedgui A, Mallat Z: In vivo induction of endothelial apoptosis leads to vessel thrombosis and endothelial denudation: a clue to the understanding of the mechanisms of thrombotic plaque erosion. *Circulation* 2004;109:2503–2506.
- 16 Gogly B, Naveau A, Fournier B, Reinald N, Durand E, Brasselet C, Coulomb B, Lafont A: Preservation of rabbit aorta elastin from degradation by gingival fibroblasts in an ex vivo model. *Arterioscler Thromb Vasc Biol* 2007;27:1984–1990.
- 17 Yamaguchi T, Yokokawa M, Suzuki M, Higashide S, Katoh Y, Sugiyama S, Misaki T: Factors influencing mortality in the rat elastase-induced-aneurysm model. *J Surg Res* 2000;94:81–83.
- 18 Allaire E, Forough R, Clowes M, Starcher B, Clowes AW: Local overexpression of TIMP-1 prevents aortic aneurysm degeneration and rupture in a rat model. *J Clin Invest* 1998;102:1413–1420.
- 19 Yamaguchi T, Yokokawa M, Suzuki M, Higashide S, Katoh Y, Sugiyama S, Misaki T: Morphologic changes in the aorta during elastase infusion in the rat aneurysm model. *J Surg Res* 2001;95:161–166.