Embolization of a common carotid aneurysm with rhVEGF coupled to a pH-responsive chitosan in a rat model

Laboratory investigation

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Object. Treatment of cerebral aneurysms by endovascular deployment of liquid embolic agents has been proposed as an alternative strategy to conventional coiling, and new materials are being developed for embolization. In this study, the authors used a single-injection, biocompatible, biodegradable and pH-responsive acrylated chitosan (aCHN) with conjugated vascular endothelial growth factor (rhVEGF) in a rat aneurysm model.

Methods. The efficacy of the aCHN formulation with rhVEGF was tested using a common carotid artery occlusion model in rats, and the extent of embolization was evaluated using quantitative, qualitative, and histopathological techniques after 14 days of implantation.

Results. The mean occlusion was significantly greater for the rhVEGF/aCHN-treated group (96.8 ± 3.0%) than for the group receiving aCHN (74.7 ± 5.6%) (p < 0.01). Through qualitative evaluation, intimal and medial proliferation were significantly greater with rhVEGF/aCHN than with aCHN and controls (p < 0.001). Degradation of the aCHN filler was monitored in concert with the production of extracellular matrix components. Macrophages migrated in and proliferated inside the occluded carotid artery lumens were identified by histological and immunostainings. Results showed resorption of chitosan with concurrent development of collagen and elastin into the vessel lumen, suggesting clot maturation into fibrosis.

Conclusions. Chitosan with a bioactive agent such as rhVEGF showed excellent results in occluding aneurysms in a rat model. (DOI: 10.3171/2009.1.JNS08411)

Key Words • intracranial aneurysm • embolization • vascular endothelial growth factor

Endovascular therapy is an evolving field with significant improvements in technology leading to more effective treatment strategies. Endovascular coils have been the mainstay of catheter-based therapy for aneurysms, and multiple studies have described modifications that may enhance their performance.1,11 However, liquid embolic agents are now of particular interest and are being evaluated for the treatment of not only arteriovenous malformations, but also cerebral aneurysms. Onyx (MTI) is composed of dimethyl sulfoxide and ethylene vinyl alcohol and has been successfully used to embolize arteriovenous malformations. It was recently approved for the treatment of intracranial aneurysms.2,15

Treatment of cerebral aneurysms by liquid embolic agents has been proposed as an alternative strategy to coiling, and new materials are being developed. In this study, we used a single-injection, biocompatible, biodegradable, and pH-responsive aCHN with rhVEGF as an experimental strategy to promote tissue filling of aneurysms. Chitosan is well known as a hemostatic and antibacterial biomaterial; VEGF is a well-known mitogen for fibroblasts, endothelial cells, smooth muscle cells, and a number of other cell types, making it an optimal agent for vascular embolization with a delivery agent such as chitosan.4 Accordingly, the efficacy of the aCHN gel formulation with rhVEGF was tested in a CA model in rats, and the extent of embolization was evaluated. Degradation of the aCHN filler was monitored in concert with the productions of ECM components. Macrophages migrated in and proliferated inside the occluded carotid artery lumens were identified by histological and immunostainings. Results showed resorption of chitosan with concurrent development of collagen and elastin into the vessel lumen, suggesting clot maturation into fibrosis.

Conclusions. Chitosan with a bioactive agent such as rhVEGF showed excellent results in occluding aneurysms in a rat model. (DOI: 10.3171/2009.1.JNS08411)

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Abbreviations used in this paper: aCHN = ampholytic chitosan; CA = carotid artery; CCA = common CA; ECM = extracellular matrix; GAG = glycosaminoglycan; PBS = phosphate-buffered saline; rhVEGF = recombinant human vascular endothelial growth factor; vWF = von Willebrand factor.

This article contains some figures that are displayed in color online but in black and white in the print edition.

658 J Neurosurg / Volume 112 / March 2010
Embolization of rat carotid artery aneurysm with modified chitosan

muscle actin and rabbit anti–rat vWF were purchased from Abcam. The secondary antibodies were from Ray-Biotech, Inc. All other chemicals were of reagent grade, and distilled and deionized water was used.

Preparation of Bioactive Ampholytic Chitosan Solution

For a typical synthesis, a method we have previously used was followed. Briefly, 3 g of chitosan was dissolved in 150 ml of 2.75% (vol/vol) aqueous acrylic acid solution; it was heated and maintained at 50°C under constant vigorous agitation for 48 hours. Upon cooling to ambient temperature, the pH of the reaction mixture was adjusted to ~11 using 10 M NaOH solution. After exhaustive dialysis for 3 days, the aCHN was recovered by lyophilization.

A 2% (wt/vol) aCHN solution was prepared by dissolving the proper amount of aCHN in water with its pH previously adjusted to ~6. A stock rhVEGF solution (250 ng/μl) was prepared by dissolving rhVEGF in sterile PBS. One hundred microliters of the rhVEGF solution was gently blended with 900 μl of the aCHN solution prepared previously with a micropipette tip to form a bioactive viscous rhVEGF/aCHN solution.9

Animal Surgery and Polymer Infusion

The animal model used was modified from a previously established procedure for adult rats.16 All animal studies were performed following the guidelines prescribed by the Institutional Animal Care and Use Committee of State University of New York-Stony Brook (IA-CUC Protocol No. 2007–1598) in compliance with the standard established in the Guide for the Care and Use of Laboratory Animals of the National Research Council. Briefly, anesthesia was induced in Sprague-Dawley rats (weight range 375–450 g) with 5% isoflurane and main-}

harvest of vessels

2O2-fibated for 10 minutes with 1% H}

Histopathological Assessment

Following standard histology processing protocols, formalin-fixed CCA segments were embedded in paraffin, sectioned, and stained with H & E. The sections were observed under a microscope (Zeiss Axiolux 200 M), and the images were captured and digitized with a camera (AxioCam MRc, Zeiss). The images were analyzed and quantified using NIH Image J software for their percentages of occlusion. The data are expressed as the means ± SDs. The Student t-test was used to determine the statistical differences between groups. Semiquantitative pathological evaluation of vessel intimal, medial, and luminal proliferation of the histological sections was performed by a single observer (J.M.A.) who was blinded to the experimental protocol by using a scale of 1–4 (a score of 4 indicates the greatest magnitude of proliferation).

The vessel specimens were stained with safranin-O/ fast green and iron-hematoxylin. Fast green staining confirmed the presence of chitosan residue, and safranin-O staining helped to visualize the existence of GAG/proteoglycan.12 The elastin and collagen fibers on the tissue section were stained with a modified Verhoeff-van Gieson method using a commercially available staining kit (EMS).10

Immunohistochemical Analysis

Briefly, all histology sections prepared were incubated for 10 minutes with 1% H2O2 in PBS to inactivate endogenous peroxidase, followed by a blocking step with 10% bovine serum albumin in PBS for 30 minutes at ambient temperature. The specific antibodies (anti-CD68 1:300, anti–vWF 1:800, and smooth muscle actin 1:0) diluted in blocking buffer were deposited on the sections for 1 hour, followed by incubation with the corresponding secondary antibody for another hour. Positive staining was revealed with a DAB kit (Vector Laboratories), and all sections were counterstained with nuclear fast red. For CD68, positive staining was shown with a fluorescein isothiocyanate–labeled secondary antibody and the sections were counterstained with DAPI (Vector Laboratories).

Results

Quantitative Analysis

The extents of vessel occlusion are summarized in Fig. 2, and the corresponding representative histological sections are depicted in Fig. 3. As evident from Fig. 3A, the group injected with rhVEGF/aCHN (11 rats) showed

Harvest of Vessels

Two weeks after performing the surgical procedure, the rats were killed with CO2. The original incision was reopened, and the previously intervened CCA segments were resected and preserved in formalin. The contralateral CCAs were excised as uninvolved controls.

J Neurosurg / Volume 112 / March 2010

659
practically complete occlusion of the arterial lumen (98.6 ± 3.0%, Fig. 3A). The vessel lumens of the group treated with aCHN alone (9 rats) showed profound intimal hyperplasia with partially filled lumens (74.7 ± 5.6%, Fig. 3B). The vessel lumens retrieved from the saline- (8 rats) or rhVEGF solution– (9 rats) treated groups showed mild to moderate intimal proliferation response (32.3 ± 11.2 and 30.6 ± 10.4%, respectively, Fig. 3C and D). When comparing vessel lumen patency, the rhVEGF/aCHN group had significantly more hyperplasia than the other groups, aCHN alone, rhVEGF solution, and saline (p < 0.01). The vessel lumens from the contralateral vessels receiving no intervention had normal appearances (results not shown).

Qualitative Analysis

The results of pathological scoring for vessel intimal, medial, and luminal proliferation (all on Grades 0–4) are summarized in Table 1. The scores of the rhVEGF/ aCHN-treated group were significantly greater than those of the other groups (saline, rhVEGF, and aCHN), which underscored the efficacy of combining the pH-responsive aCHN gel with rhVEGF (p < 0.001).

Degradation of Chitosan Gel and Production of GAGs

Safranin-O/fast green and iron-hematoxylin staining were carried out to validate the degradation of the aCHN gels inside the arterial lumens 2 weeks postinjection as well as the corresponding distribution of newly synthesized GAGs within the filled arterial lumen. Staining by safranin-O detects the presence of anionic GAG, whereas fast green has a strong affinity toward the polycationic chitosan.11 Chitosan (typically shows up as brilliant green, if stained positive) was noticeably absent in the arterial lumens injected with rhVEGF/aCHN, suggesting the complete bioresorption of the aCHN gel (Fig. 4A and B), and the rhVEGF was released by the degradation of the aCHN gel. In addition, the ingrowth tissue showed up as intense red, indicating the abundance of GAGs (Fig. 4A and B). There was an absence of entrapped erythrocytes in the ingrowth tissue, which would otherwise show up as light green. A brilliant green color appeared in the arterial lumen originally injected with aCHN (Fig. 4C and D), indicating the presence of residual aCHN. Furthermore, multiple cell layers were identified between the aCHN core and the arterial wall with neither identifiable cells nor GAGs inside the core of the residual aCHN.

Production of Elastins and Collagens

We proceeded to functionally characterize the ingrowth cells and tissues in the arterial segments originally injected with either rhVEGF/aCHN or aCHN. One important function of the cells populating the arterial lumens could presumably be producing elastin and collagen, 2 main ECM components in blood vessels; therefore, the specimens were stained with a modified Verhoeff-van Gieson method. The results depicted in Fig. 5A and B indicated that both elastin fibers (bluish black) and collagen fibers (pink) were present among the ingrowth tissue in the arterial lumens injected with rhVEGF/aCHN. The newly synthesized collagen and elastin fibers were intermingled and distributed uniformly inside the vessel lumen. Some smooth muscle cells (staining greenish yellow) were found dispersed inside the tissue-filled lumen. In contrast, the arterial lumen injected with the aCHN gel showed up as dark brown after staining, indicating the absence of both collagen and elastin fibers, which further suggested that cells did not penetrate the aCHN core (Fig. 5C and D).

Macrophage Staining

Macrophage is the most important cell type, playing an active role both in removal of implanted material and inflammatory responses. The marker for rat macrophag-
es and monocytes is CD68; therefore, all samples were stained with anti-CD68 antibody. In the arteries injected with rhVEGF/aCHN, a great number of macrophages (green) were detected in the ingrowth tissue (Fig. 6A and B). Although both elastin and aCHN autofluoresced (appearing as green), DAPI counterstaining did not identify any cell present inside the aCHN; however, there were noticeable cells present in between the aCHN residue and the vessel wall (Fig. 6C and D), suggesting that macrophages only surrounded but did not deeply penetrate the aCHN residue. Figure 6E and F revealed the stained monocytes/macrophages (trapped along with blood) in control samples.

von Willebrand Factor Staining

Endothelial cell specific anti–vWF was used to identify endothelial cells in the ingrowth tissue. Figure 7D showed that only the innermost layers of cells toward the lumen stained positively in the control artery. In Fig. 7A (on the edge of the artery lumen specimen) and 7B (inside the lumen of the artery specimen), endothelial cells formed capillaries inside the ingrowth tissue in the specimen treated with the rhVEGF/aCHN gel, and no endothelial cells were found in the arteries injected with aCHN alone (the aCHN appeared dark brown after staining). In addition, cells between the artery wall and the aCHN residue stained negative, further implicating the absence of endothelial cells.

Smooth Muscle Actin Staining

Smooth muscle actin is a specific indicator of smooth muscle cells; therefore, anti–smooth muscle actin antibody was used to verify the presence of smooth muscle cells in the ingrowth tissue (Fig. 8). Smooth muscle cells were identified between layers of elastin fibers in the artery wall of the control specimens (Fig. 8D). In contrast, smooth muscle cells were distributed evenly inside the ingrowth tissue in the samples injected with rhVEGF/aCHN (Fig. 8A [edge] and 8B [inside of the lumen]). Likewise, smooth muscle cells were found in the arterial lumen between the artery wall and the aCHN (Fig. 8C).

Discussion

Many growth factors, such as basic fibroblast growth factor, transforming growth factor, and VEGF, have been reported to enhance fibrotic tissue formation and endothelialization in in vivo studies; in general, they were coupled to embolic agents as experimental treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of Rats</th>
<th>Intimal</th>
<th>Medial</th>
<th>Luminal</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>10</td>
<td>1.0 ± 0.0</td>
<td>1.1 ± 0.3</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>saline</td>
<td>8</td>
<td>1.4 ± 0.5</td>
<td>1.4 ± 0.7</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>aCHN</td>
<td>9</td>
<td>3.1 ± 0.6</td>
<td>3.0 ± 0.0</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>rhVEGF</td>
<td>9</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.4</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>rhVEGF/aCHN</td>
<td>11</td>
<td>4.0 ± 0.0</td>
<td>3.8 ± 0.4</td>
<td>3.9 ± 0.3</td>
</tr>
</tbody>
</table>

* The statistical differences between the group that received rhVEGF/aCHN and other groups are all < 0.01.
for aneurysms; nonetheless, complete occlusion had not been reported.\textsuperscript{5,6,14,16} Vascular endothelial growth factor is produced by macrophages, endothelial cells, and smooth muscle cells and appears to be an optimal agent for occlusion of aneurysms or any vascular structure.\textsuperscript{4} Vascular endothelial growth factor is a well-documented mitogen for fibroblasts, endothelial cells, smooth muscle cells, and a number of other cell types.\textsuperscript{4} In particular, it is highly potent in stimulating collagen production leading to the formation of fibrotic tissue in the vessel lumen and neointimal closure.\textsuperscript{14,16} Due to VEGF’s short half-life in vivo, conventional application of VEGF solution exerts virtually no biological effects.

In this investigation, we have formulated an experimental liquid embolic agent by coupling rhVEGF to a catheter-deployable, pH-responsive chitosan-based polymeric system. On deployment, this agent is capable of conforming to the vessel lumen and is rendered insoluble at physiological pH, thereby embolizing and completely filling the vessel lumen. Native chitosan is a crustacean-
Embolization of rat carotid artery aneurysm with modified chitosan

derived polycationic carbohydrate produced by deacetylation of chitin and is very abundant in nature. Chitosan is biocompatible and biodegradable, with a high degree of bioadhesiveness to tissues, and it is also known to be hemostatic. Chitosan could be formulated into films or scaffolds suitable for topical applications such as tissue repair, and it could also serve as a structural material for a number of tissue engineering–related applications.

As a polycation, chitosan could electrostatically conjugate delicate bioactive agents (for example, proteins, such as VEGF) that are typically anionic, while preserving their bioactivities through enhancement of stabilities.

Fig. 6. Photomicrographs. Carotid arteries injected with rhVEGF/aCHN (A and B), aCHN only (C and D), and saline (E and F). Nuclei appear blue; aCHN, bright green; and elastin, subdued green. Red arrows indicate ingrowth tissue; asterisks, aCHN residue; and yellow arrows, the artery wall. Anti-CD68; bar = 200 µm (A), 100 µm (C, E, and F), and 50 µm (B and D).

Fig. 7. Photomicrographs. Carotid arteries injected with rhVEGF/aCHN (A and B), aCHN only (C), and saline (D). The boundary of artery wall and vessel lumen (A, C, and D) and ingrowth tissue in the lumen (B) are seen. Endothelial cells appear dark brown; nuclei, pink; and aCHN, light brown. Asterisk denotes aCHN; black arrows, endothelial cells; and yellow arrows, the artery wall. WAnti–vWF antibody; bar = 50 µm.
In contrast to most other hydrogel materials that are not suitable for microcatheter delivery and thus, could not be applied to address aneurysms, aCHN solution precipitates at physiological pH; this feature enables it to traverse a microcatheter and forms sediment in an aneurysm sac. We have also previously demonstrated the feasibility of using aCHN as a mechanism for controlled release of protein.9

As shown in Fig. 3A, application of the rhVEGF/aCHN combination resulted in a profound but localized response, leading to complete filling of the vessel lumen by a robust fibrotic tissue development. Interestingly, application of the aCHN alone also resulted in an intense response but of significantly lower magnitude, as manifested by the tissue development (Fig. 3B). This effect was likely induced by a combination of the inflammatory reaction through the presence of aCHN and the stenotic response to arterial injury after polymer infusion.

The notion of an arterial injury–induced stenotic-type response was substantiated by the moderate tissue proliferation produced after the infusion of saline and rhVEGF solutions, respectively (Fig. 3C and D). Nonetheless, the stenotic response alone could not completely account for the profound tissue development observed in the vessels treated with rhVEGF/aCHN. In the samples injected with rhVEGF/aCHN, the aCHN apparently fully degraded in the 2-week time span (Fig. 4A and B), whereas other embolic agents were reported to take considerably longer to degrade (12 weeks or even longer).18

The consistent presence of a small amount of rhVEGF released by gradual degradation of the aCHN evidently resulted in stimulation of robust tissue development (Fig. 4A and B); this in turn increased the metabolic activity occurred in the arterial lumen, thus hastening the aCHN degradation compared with samples injected with aCHN alone (Fig. 4C and D).

Disruptions in the turnover and organization of both collagen and elastin are proposed as the leading contributing factors in initiating aneurysm sac formation.8 Endovascular embolic agents made from biodegradable materials could serve as transient platforms for tissue development, leading to a complete and permanent occlusion of an aneurysm with mature connective tissue. Histological staining of the specimens demonstrated intense ECM deposition and cellular infiltration into the vessel segments embolized with rhVEGF/aCHN. By Day 14, the rhVEGF delivered by aCHN had apparently induced the formation of mature fibrotic tissue inside the vessel lumens with dominant collagen/elastin/GAG deposits (Fig. 4A and B). Deploying the rhVEGF/aCHN to aneurysm sacs could result in comparable tissue growth, reinforcing the weakened arterial wall, thus minimizing the possibility of recurrence.

A complementary mode of preventing aneurysm recurrence after embolization is to induce thrombus organization in the aneurysm through promoting the migration and proliferation of vascular cells into the lesion. Biodegradable materials are expected to stimulate cellular infiltration and proliferation during their degradations. The results from immunostaining the specimens depicted in Figs. 6–8A and B indicated that macrophages, endothelial cells, and smooth muscle cells (also in Fig. 5A and B) were present in the ingrowth tissue of the vessel lumens injected with rhVEGF/aCHN, and these 3 cell types are known to express receptors for VEGF.4

Macrophages modulated the inflammatory responses
toward the implanted aCHN, which was foreign to the local tissue. They also played a significant role in degradation and eventually removal of the aCHN. Importantly, no foreign body giant cell was observed on the specimen, indicating the absence of chronic inflammatory responses. Mature capillaries formed by endothelial cells were evidently present in the vessel lumen of the artery injected with rhVEGF/aCHN (Fig. 7A and B); they serve the function of sustaining the hemodynamic needs of the ingrowth tissue and prevent tissue necrosis from occurring. It is probable that other types of cells, such as fibroblast cells, could also be present in the tissue-filled vessel lumen; unfortunately, detection of fibroblast is currently not feasible due to the unavailability of markers specific to rat fibroblasts. Nonetheless, some macrophages and smooth muscle cells were detected between the aCHN residue and the artery wall, but they were noticeably absent inside the aCHN remnants (Figs. 6C and D and 7C and 8C), implying that the aCHN alone had very limited bioactive functions.

A major limitation of this study is the small animal model used to extrapolate vascular biology. The model is a CA stump model rather than a CA “aneurysm” model, and larger animals would be necessary to confirm the same results. Another limitation of the study is the expected interaction of chitosan and polymerization with the CA or cerebral blood flow. The modified chitosan we used in this study was not intended for polymerization; the mechanism for embolization was precipitation. We have since been using an improved version of the modified chitosan for our large animal studies, and a nontoxic macromolecular crosslinker was included to facilitate rapid polymerization. Another limitation with any liquid embolic is adhesiveness to the catheter. Since the completion of this study, we have modified the formulation and used it in larger animal models without any adhesiveness to the catheter exterior. The catheter is easily removed once the biomaterial is deployed.

Conclusions

The combination of rhVEGF and aCHN solution has shown promising results in a small animal model. This treatment is bioresorbable, it has no apparent toxicity to surrounding tissues upon deployment, and it is capable of inducing endogenous tissue ingrowth with newly synthesized ECM and capillaries. Current investigation is focused on validating its efficacy and long-term effects in large animal models.

Disclosure

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References


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