Low molecular weight (LMW) heparin inhibits injury-induced femoral artery remodeling in mouse via upregulating CD44 expression

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Objective: **The mechanism of postangioplasty restenosis remains poorly understood. Low molecular weight (LMW) heparin has been shown to inhibit the proliferation of vascular smooth muscle cells (VSMCs), which is the principal characteristic of restenosis. Studies have shown that LMW heparin could bind to CD44. We hypothesized that LMW heparin might modulate CD44 expression thereby decreasing vascular remodeling.**

Methods: **Vascular remodeling was induced in $CD44^{+/+}$ and $CD44^{-/-}$ mice and treated with LMW heparin. The arteries were harvested for histologic assessment and determination of CD44 expression. Bone marrow transplantation was introduced to further explore the role and functional sites of CD44. Effects of LMW heparin on growth capacity, CD44 expression were further studied using the cultured mouse VSMCs.**

Results: **Transluminal injury induced remarkable remodeling in mouse femoral artery (sham wall thickness percentage [WT%]: 3.4 ± 1.2% vs injury WT%: 31.8 ± 4.7%; $P < .001$). LMW heparin reduced the remodeling significantly (WT%: 17.8 ± 3.5%, $P < .005$). $CD44^{-/-}$ mice demonstrated considerably thicker arterial wall remodeling (WT%: 46.2 ± 7.6%, $P = .0035$), and CD44-chimeric mice exhibited equal contributions of the local and circulating CD44 signal to the neointima formation. LMW heparin markedly upregulated CD44 expression in the injured femoral arteries. In vitro, LMW heparin decreased mouse VSMC growth capacity and upregulated its CD44 expression simultaneously in a dose-dependent and time-dependent manner, which could be partially blocked by CD44 inhibitor.**

Conclusions: **LMW heparin inhibits injury-induced femoral artery remodeling, at least partially, by upregulating CD44 expression. (J Vasc Surg 2011;53:1359-67.)**

Clinical Relevance: **Angioplasty is widely used in clinical practice to treat various stenostic vascular disorders, but the postangioplasty reocclusion has been a big limit and the mechanism underlying the vascular remodeling remains poorly understood. LMW heparin has been a promising medicine to inhibit VSMC proliferation. However, the mechanism of LMW heparin inhibition against smooth muscle cell (SMC) proliferation and its clinical usefulness is still not clear. Our present data, which were based on in vivo and in vitro studies, suggested that LMW heparin induced higher CD44 expression in VSMCs, and through the CD44 pathway, LMW heparin significantly reduced SMC proliferation and injury-induced femoral artery remodeling. Our study clarified the roles of LMW heparin in vascular occlusive diseases. This study helps to elucidate the underlying cellular and molecular mechanism by which LMW heparin inhibits injury-induced remodeling and could promote creating new therapeutics to control the exaggerated neointimal hyperplasia.**

Angioplasty is a commonly used treatment for various occlusive lesions. However, there are many risks associated with this treatment. One such risk is restenosis. Restenosis after angioplasty has been a major limitation in the long-term prognosis of this treatment. Although much effort has been devoted to understanding this complication, the mechanism underlying the remodeling of the arterial vasculature remains poorly understood.[1,2] One principal characteristic of restenosis is the prominent accumulation of vascular smooth muscle cells (VSMCs). VSMCs have been one of the key targets in prevention and treatment of occlusive vessel diseases.[3]

Low molecular weight (LMW) heparin is a glycosaminoglycan that is clinically used as an antithrombotic agent. LMW heparin has been shown to inhibit VSMC proliferation.[4] Our previous studies have indicated that LMW heparin inhibits pulmonary vascular remodeling in bovine and rodent models,[5,6] but the mechanism of this inhibition is not well understood.

CD44 is a specific cell-surface receptor for hyaluronan. Previous studies have revealed that it is one of the target genes of high-mobility-group proteins, which plays a major

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role in the development of atherosclerotic lesions, characterized by VSMC proliferation.[7,8] Kothapalli found that CD44 showed antimitogenic effects and was associated with altered VSMC proliferation.[9] Harris documented that free LMW heparin could bind to hyaluronan and CD44 to form a compound.[10] However, there are few studies defining the role of CD44 in arterial proliferative lesion, and it is not clear whether LMW heparin modulates CD44 expression in vascular remodeling.

We developed an injury-induced vascular remodeling model by temporally placing a spring guidewire into a mouse femoral artery. Using this model, we designed the present study to understand the roles of LMW heparin and CD44 in arterial remodeling, as well as the effects of LMW heparin on CD44 expression.

METHODS AND MATERIALS

Animals. Six- to 8-week-old inbred male CD44 knockout mice ($CD44^{-/-}$, Genotype: CD44 ^{tm1Hbg} /J) and wild-type control mice ($CD44^{+/+}$, C57BL/6J), weighing 21 to 25 g, were obtained from the Jackson Laboratory, Bar Harbor, Me. All the procedures were performed in accordance with protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, Harvard University and in compliance with the "Guide for the Care and Use of Laboratory Animals" (Publication No. NIH 78-23, 1996). The $CD44^{-/-}$ mice used in the present work were viable, fertile, and normal in size. They did not display any gross physical or behavioral abnormalities. No CD44 gene products (mRNA or protein) were detected in these mice.

Mouse transluminal injury-induced femoral artery remodeling. Mice were randomly placed into various experimental groups ($CD44^{+/+}$ sham; $CD44^{+/+}$ injury; $CD44^{+/+}$ injury/heparin; $CD44^{+/+}$ injury/saline; $CD44^{+/+}$ injury/warfarin; $CD44^{-/-}$ sham; $CD44^{-/-}$ injury; $CD44^{-/-}$ injury/ heparin). Five mice were used in each group. To achieve a surgical level of anesthesia, the mice were anesthetized by intraperitoneal injection of 50 mg/kg ketamine hydrochloride diluted in 0.9% sodium chloride solution. The surgical procedure used was a simplified version of one previously described by Masataka Sata and Ernane Reis.[11,12] Briefly, a groin-to-knee incision was made in the left hind leg and the femoral artery was dissected. Through a muscular branch between the rectus femoris and vastus medialis muscles, a spring guidewire (0.38 mm in diameter, No. C-SF-15-15; Cook, Bloomington, Ind) was gently advanced into the femoral artery at least 6 mm in-depth and kept in place for 20 seconds. The femoral artery blood flow was restored after the surgery. The mice were euthanized at fixed endpoints, and the femoral arteries underwent perfusion fixation by flushing 0.9% sodium chloride solution followed by 10% paraformaldehyde buffer (pH 7.4) through the left ventricle. The vessels were then carefully harvested and postfixed in 10% paraformaldehyde buffer (pH 7.4).

A time course of remodeling was investigated by harvesting the femoral arteries from $CD44^{+/+}$ mice at weekly time points, week 1 through week 7 postsurgery. There were five mice in each time point group.

We determined the dose of LMW heparin by titration to achieve the most effective action while avoiding its toxicity. As previously described,[6] the mice under heparin treatment (five mice included in each group) were given 20 mg/kg of LMW heparin (heparin sodium salt; MP Biomedicals, LLC, Solon, Ohio) subcutaneously once a day for 4 weeks. The first loading dose of 40 mg/kg was administered subcutaneously 1 hour prior to the surgery. In the sham group (five mice were included), the surgery was performed without transluminal injury. The purity of LMW heparin was tested by capillary electrophoresis and isoelectric focusing/two-dimensional electrophoresis by MP Biomedicals, LLC to exclude any contamination, and the purity was found to be 99.8%.

To determine whether the antiproliferative action of LMW heparin was due to its anticoagulant property, we compared the effect of warfarin (Coumadin; DuPont Pharmaceuticals, Wilmington, Del), a thrombin inhibitor, given at an anticoagulant dose of 0.25 mg/kg/d subcutaneously (SC) with a loading dose of 0.5 mg/kg/d for 3 days prior to the surgery to LMW heparin. We established a dose of warfarin that would exhibit equal ability as LMW heparin to inhibit thrombin generation determined by prothrombin time (PT) test. Mice treated with warfarin (0.25 mg/kg/d SC) or LMW heparin (20 mg/kg/d SC) presented an identical increase in PT from the normal range of 10 ± 3 to 45 ± 8.3 seconds without any accompanying toxicity.

Elastin Van Gieson staining and histopathologic analysis. The perfusion-fixed mouse common femoral arteries were cut into two 2- to 3-mm segments for paraffin embedding. Multiple cross-sections (5 μm) of both segments underwent elastin Van Gieson staining of the internal elastic lamina for accurate measurement of the neointima. Each group included at least five femoral arteries, and any arteries which appeared to contain thrombi were excluded from the evaluation. Digitized images were analyzed with image analysis software ImageJ v. 1.33 u (NIH) by two blinded investigators independently. Mean remodeling values were obtained via averaging the values from each animal in the same group. The technique used to measure wall thickness percentage (WT%) is described in the supplement section (Appendix, online only) of this article. For the histologic assessment, five mice were used in each group under various experimental conditions.

Western blot analysis. Western blot analysis was performed as previously described.[5] Protein was extracted from the femoral arteries of each group (five mice were used in each group) or cultured mouse VSMCs. CD44 antibody (rabbit polyclonal antibody; Abcam Inc, Cambridge, Mass) and GAPDH (mouse monoclonal antibody, Research Diagnostics Inc, Flanders, NJ) were used for Western blot detection.

Bone marrow transplantation. To further elucidate whether CD44 was involved and to examine the contributive sites of CD44 signaling in the neointima formation, we established CD44 chimeric mice by bone marrow trans-

plantation. Bone marrow cells were harvested from femurs and tibias of female $CD44^{+/+}$ or $CD44^{-/-}$ mice. Femoral artery injuries were performed 6 weeks after the bone marrow transplantation. Two types of chimeric mice were created: $CD44^{+/+}$ mice with $CD44^{-/-}$ bone marrow (KO-WT) and $CD44^{-/-}$ mice with $CD44^{+/+}$ bone marrow (WT-KO). $CD44^{-/-}$ with $CD44^{-/-}$ bone marrow (KO-KO) and $CD44^{+/+}$ with $CD44^{+/+}$ bone marrow (WT-WT) were prepared as control groups at the same time. In every group, one mouse was used as bone marrow donor, and transplantations were performed on five receipt mice. We harvested the femoral arteries from the receipts 4 weeks postsurgery for the histologic assessment.

Mouse vascular smooth muscle cell culture. Having not found any difference between mouse thoracic/abdominal aorta and femoral artery VSMCs, we chose to isolate thoracic and abdominal aorta VSMCs from the mice of each group (five mice per group). Isolation of the VSMCs was done by scraping the endothelium and removing the adventitia of the arteries microscopically. The media was washed twice with reduced-Ca^{2+} HBSS. The media was cut into small pieces and then digested in reduced-Ca^{2+} HBSS containing collagenase (1750 U/mL), papain (9.5 U/mL), bovine serum albumin (2 mg/mL), and dithiothreitol (1 Mm/mL) at 37°C for 10 minutes. Then, the cells were seeded and cultured in smooth muscle basal medium with 10% fetal bovine serum (FBS; Vitromex, Vilshofen, Germany), streptomycin, 100 μg/mL (Invitrogen Life Technologies, Carlsbad, Calif) and penicillin, 100 μg/mL (Invitrogen Life Technologies).

Growth inhibition analysis on cultured VSMCs. As previously described,[5] growth inhibition analysis was introduced to assess the most effective antiproliferative dose of LMW heparin for in vitro assay. VSMCs between passages four and six were used to perform this study. The $CD44^{+/+}$ VSMCs were seeded at 1.25×10^4 cells per well in 6-well tissue culture plates and grown for 2 days in regular medium (10% FBS). The cells were then divided into three groups. Group one, the negative control, included cells cultured in 10% FBS. Group two included cells cultured in 0.1% FBS and used as a positive control. Group three included cells cultured in 10% FBS at various concentrations of LMW heparin (10, 25, 50, 100, 150, and 200 μg/mL) to determine the most effective antiproliferative dose of LMW heparin. All groups were cultured for 5 days, with the medium being changed daily. The cells were harvested and stained with Trypan blue and counted using a hemocytometer. Each group included five wells of cells. The experiment was repeated three times. The method used to calculate the percentage of growth inhibition (% inhibition) is presented in the supplement section (Appendix, online only). Five mice were used as VSMC donors.

Growth curves analysis on cultured VSMCs. Growth curve analysis was introduced to study the antiproliferative action of LMW heparin on the cultured mouse VSMCs ($CD44^{+/+}$ and $CD44^{-/-}$). VSMCs between passages four and six were used to perform this study. We performed growth curve analysis on $CD44^{+/+}$ VSMCs cultured in standard medium (10% FBS) and standard medium with 100 μg/mL LMW heparin, standard medium with 6 μg/mL anti-CD44 mAb (BD Biosciences, Franklin Lakes, NJ), and standard medium with 100 μg/mL LMW heparin as well as 6 μg/mL anti-CD44 mAb. Similarly, growth curve analysis on $CD44^{-/-}$ VSMCs was performed in standard medium (10% FBS) and standard medium with 100 μg/mL LMW heparin. Cells from five wells of each group were harvested and stained with Trypan blue and counted daily using a hemocytometer. The experiment was repeated three times. Five mice were used as VSMC donors.

Flow cytometry detection of CD44 expression on cultured VSMCs. VSMCs between passages four and six were used to perform this study. Mouse smooth muscle cells (SMCs) were seeded at 1.25×10^4 cells per well in 6-well tissue culture plates and allowed to grow for 2 days. The cells were then growth-arrested in 0.1% FBS for 48 hours and cultured for 5 days in standard medium (10% FBS) containing 100 μg/mL LMW heparin. The medium was replaced daily. CD44 expression on VSMCs was detected by one-color flow cytometry analysis. Five mice were used as VSMC donors.

Time course of CD44 expression on cultured VSMCs. A time course study was performed to evaluate the effect of LMW heparin on CD44 expression in vitro. VSMCs between the four to six passages were seeded at 1.25×10^4 cells per well in 6-well tissue culture plates, allowed to grow for 2 days, then growth-arrested in 0.1% FBS for 48 hours and then cultured in standard medium with 100 μg/mL LMW heparin for 5 days. The medium was replaced daily and the expressions of CD44 mRNA and protein were detected daily. Five mice were used as VSMC donors.

Statistical analysis. Data were presented as mean ± SEM and analyzed by one- and two-way analysis of variance. Individual group means were compared with Student's unpaired *t* test. The statistical threshold was set at $P < .05$.

RESULTS

Transluminal injury induced neointima lesion and LMW heparin inhibited the remodeling in CD44 +/+ mice. Transluminal injury induced significant remodeling in mouse femoral arteries, which was characteristic of neointima formation. After harvesting the vessels, we cut them into two 2- to 3-mm segments and embedded, respectively. The resultant data of the two segments were averaged to obtain the mean remodeling values. The injured parts were included in the samples and the two segments of one sample presented very similar thickness in remodeling. We occasionally encountered some thrombi in some mice inducing mild remodeling in the involved arteries. The total incidence was <5%. We excluded these samples from our evaluation. The time course study revealed that the neointima was first noticeable 1 week after injury and reached a peak thickness in the fourth week (Fig 1, *A*). We investigated the remodeling for up to 12 weeks postinjury and

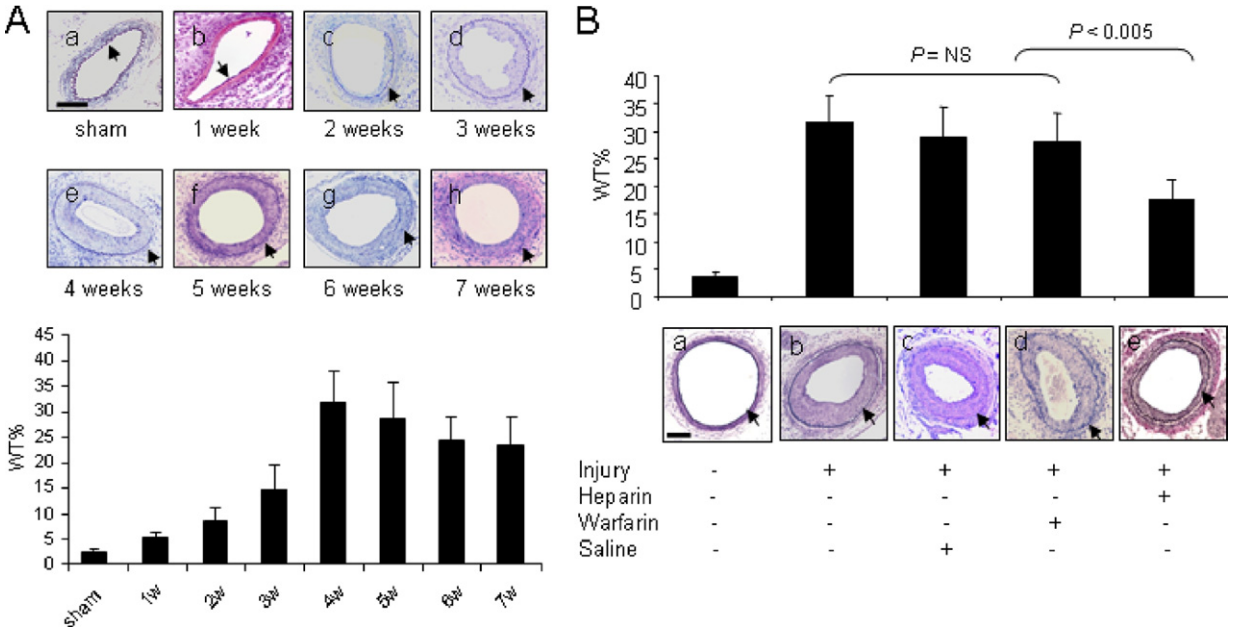

Fig 1. Time course of injury-induced femoral arterial remodeling and the inhibition of low molecular weight (*LMW*) heparin on remodeling lesion in CD44 +/+ mice. Transluminal injury induced marked remodeling in CD44$^{+/+}$ mice. The neointima emerged at the end of the first week and peaked in the fourth week after the injury (**A,** *a. sham femoral artery; b. one week after injury, HE staining, arrow indicates a thin neointima lying on the internal elastic lamina; c, d, e, f, g, and h represent remodeling of 2 weeks through 7 weeks after the injury, respectively. Elastin van Gieson staining, arrows indicate internal elastic laminas. Bar 5 μm*). In contrast with the untreated, saline-treated and warfarin-treated CD44 +/+ mice, LMW heparin reduced the wall remodeling significantly, indicating an inhibitory role in the process of vascular remodeling (**B,** *Elastin van Gieson staining, arrows indicate internal elastic laminas. Bar 5 μm*). Each group, $n = 5$.

found that after 4 weeks, the vascular wall remodeling was quite stable at a milder remodeling level.

Hence, we chose the end of the fourth week as the end time point to assess histologic changes. LMW heparin-treated CD44 +/+ mice, which received transluminal injury surgery, showed a remarkable reduction in remodeling (Fig 1, *B*). By contrast, injured arteries treated with warfarin or saline did not show any changes in remodeling thickness, indicating that the beneficial effect of LMW heparin on injury-induced vascular remodeling might not be related to its anticoagulant property.

Involvement of CD44 in remodeling. Compared with injured femoral arteries in CD44 +/+ mice, the CD44 −/− mice showed significantly thicker femoral vascular remodeling ($P = .0035$), which indicates the involvement of the CD44 gene in neointima formation. This suggests that CD44 might play an inhibitory role in the pathogenesis and development of injury-induced femoral remodeling in mice (Fig 2, *A*).

Bone marrow transplantation was introduced to further determine the role of CD44 as well as the contributive sites of CD44 signaling to the development of neointima. We documented that CD44$^{-/-}$mice given CD44$^{-/-}$ marrow (KO-KO, positive control) demonstrated markedly thicker arterial remodeling (WT%: 32.4 ± 5.5%) than that in CD44$^{+/+}$ mice given CD44$^{+/+}$ bone marrow (WT-WT, negative control; WT%: 17.7 ± 1.9%; $P = .0013$), indicating the involvement of CD44 signaling in the remodeling process. CD44$^{-/-}$ mice given CD44$^{+/+}$ bone marrow (WT-KO) presented significantly thicker vascular remodeling (WT%: 23.2 ± 4.6%) when compared with that in WT-WT mice ($P = .009$), suggesting the contribution of local CD44 signaling to neointima formation. Similarly, statistically thicker vascular remodeling in CD44$^{+/+}$ mice given CD44$^{-/-}$ marrow (KO-WT, WT%: 21.6 ± 3.7%) was recorded when compared with that in WT-WT mice ($P = .024$), suggesting the contribution of circulating CD44 to the remodeling formation. Both groups of chimeric mice (WT-KO and KO-WT) had statistically thinner remodeling compared with KO-KO mice ($P < .005$), which further demonstrated the contributions of both local and circulating CD44. The WT-KO and KO-WT chimeric groups did not differ in neointima thickness ($P = .266$) indicating the equal contribution of local and circulating CD44 to the neointima formation (Fig 2, *B*).

LMW heparin upregulated CD44 expression in remodeled femoral arteries. Compared with the uninjured arteries, the remodeled femoral arteries did not show any appreciable change in CD44 expression. Whereas, when they were treated with LMW heparin, the injured arteries demonstrated a remarkable reduction in neointima thickness and a significant increase in CD44

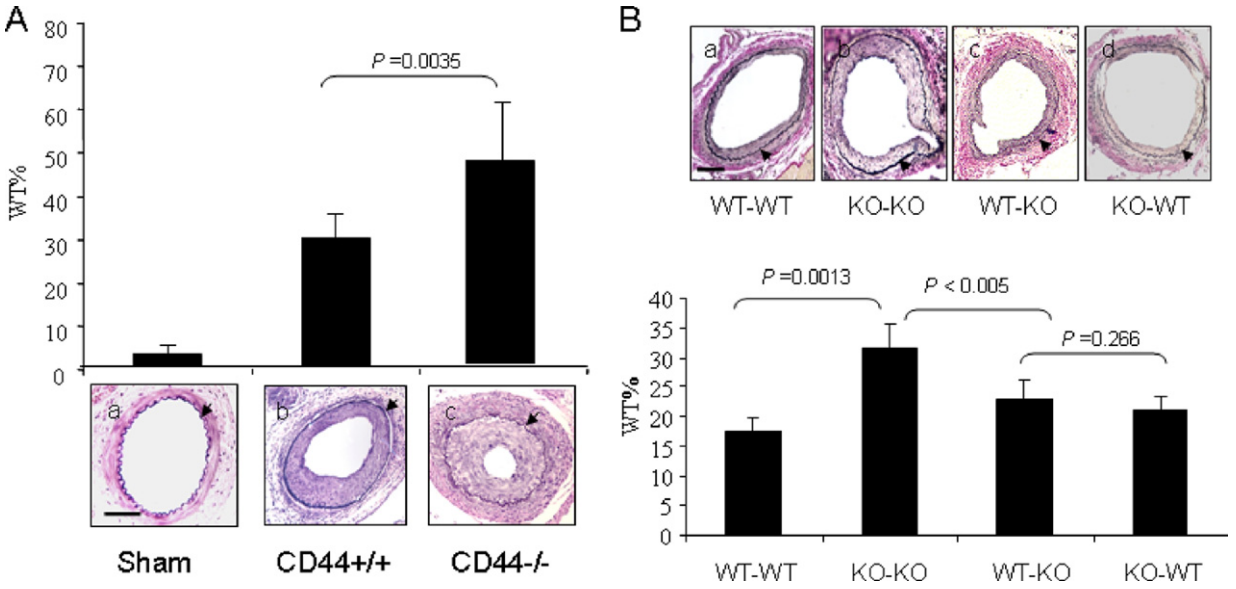

Fig 2. Involvement of CD44 in the remodeling and the contributive sites of CD44 signal to neointima formation. Postinjury remodeling in CD44 −/− mouse femoral arteries was significantly thicker than that in CD44 +/+ mice, indicating the involvement and an inhibitory role of CD44 gene in the development of the remodeling lesion. (**A:** *Elastin van Gieson staining; a, b, and c represent sham, CD44$^{+/+}$ and CD44$^{-/-}$ femoral arteries, respectively. Arrows indicate internal elastic laminas. Bar 5 μm*). CD44$^{-/-}$ mice given CD44$^{+/+}$ marrow (*WT-KO*) had significantly increased remodeling compared with CD44$^{+/+}$ mice given CD44$^{+/+}$ bone marrow (*WT-WT, negative control. P = .0013*). Statistically thicker vascular remodeling in CD44$^{+/+}$ mice given CD44$^{-/-}$ marrow (*KO-WT*) was recorded when compared to WT-WT. Both chimeric groups (*WT-KO and KO-WT*) have much thinner remodeling compared to CD44$^{-/-}$mice given CD44$^{-/-}$ marrow (*KO-KO, positive control. P < .005*). The WT-KO and KO-WT chimeric groups did not differ in neointima thickness (*P* = .266), indicating the equal contribution of local and circulating CD44 signal to the neointima formation (**B,** *Elastin van Gieson staining, a, b, c and d represent WT-WT, KO-KO, WT-KO, and KO-WT femoral artery, respectively. Arrows indicate internal elastic laminas. Bar 5 μm*). Each group, *n* = 5.

expression (Fig 3). This suggested CD44 might be a pathway by which LMW heparin decreased the remodeling. By contrast, warfarin or saline-treated injured femoral arteries did not show any changes in remodeling thickness or CD44 expression.

LMW heparin did not inhibit the vascular remodeling in CD44−/− mice. When LMW heparin was administrated to the injured CD44$^{-/-}$ mouse, we found no significant reduction in neointima formation compared with the untreated CD44$^{-/-}$ femoral arterial neointima (42.7 ± 4.1% vs 46.2 ± 7.6%, $P > .05$). By contrast, the LMW heparin-treated CD44$^{+/+}$ mice showed a significant reduction in remodeled wall thickness (18.6 ± 4.6%, $P < .005$). These histologic results suggest that LMW heparin exerts its antiproliferative effect on the vascular remodeling lesion, at least partially, via CD44 signal pathway (Fig 4).

LMW heparin did not inhibit the growth of cultured CD44 −/− VSMCs. LMW heparin was administered at different doses to cultured CD44$^{+/+}$ VSMCs. Based on the inhibition analysis, we found that LMW heparin inhibited mouse VSMCs growth in a dose-dependent manner. At increasing LMW heparin concentrations, the growth capacities of the cultured VSMCs decreased, respectively. Much higher concentrations of LMW heparin, 150 and

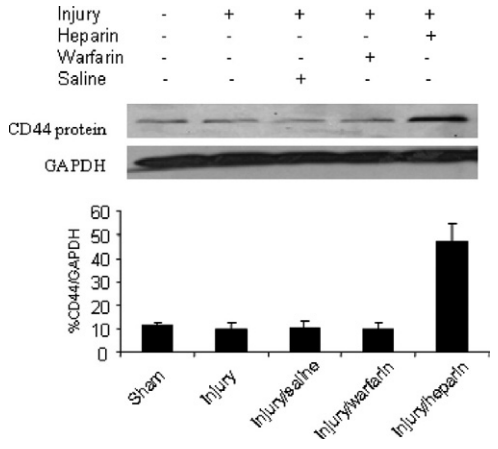

Fig 3. Low molecular weight (*LMW*) heparin upregulated CD44 expression in the injured CD44 +/+ femoral arteries. In CD44$^{+/+}$ mice, the injured arteries did not show a significant change of CD44 expression compared with the uninjured CD44$^{+/+}$ mice. When treated with LMW heparin, the injured femoral arteries expressed much higher CD44 protein (*Western blot detection of CD44 protein*) in the fourth week after the injury, whereas warfarin or saline did not affect the expression of CD44. *GADPH*, Glyceraldehyde-3-phosphate dehydrogenase.

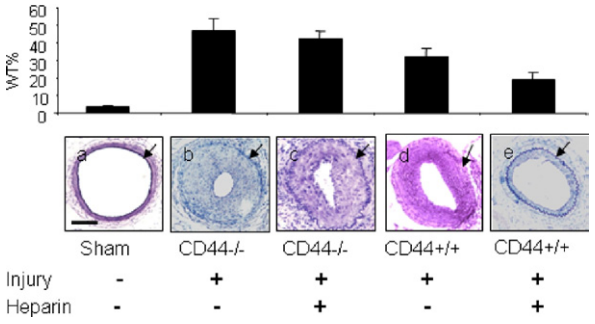

Fig 4. Effects of low molecular weight (*LMW*) heparin on the remodeling of $CD44^{-/-}$ femoral arteries In injured $CD44^{-/-}$ mice, LMW heparin did not inhibit the remodeling compared with the non-LMW heparin-treated $CD44^{-/-}$ femoral arteries (*$CD44^{-/-}$ vs $CD44^{-/-}$/LMW heparin; $P > .05$*). LMW heparin-treated $CD44^{+/+}$ mice exhibited significant reduction in remodeling (*$CD44^{-/-}$/LMW heparin vs $CD44^{+/+}$/LMW heparin $P < .001$; $CD44^{+/+}$ vs $CD44^{+/+}$/LMW heparin $P < .005$*), indicating the possible CD44 pathway through which LMW heparin inhibited the remodeling. (*Elastin van Gieson staining. Arrows indicate internal elastic laminas. Each group, $n = 5$*).

200 μg/mL, were noticed to induce death in a fraction of VSMCs (the inhibition percentages were 112.5% and 134.2%, respectively), and, hence, 100 μg/mL was determined as the optimal concentration for in vitro assay (Fig 5, *A*).

We compared the growth capacity of LMW heparin-treated $CD44^{-/-}$ VSMCs to that of the untreated $CD44^{-/-}$ VSMCs. No significant reduction in growth capacity was observed in the LMW heparin-treated $CD44^{-/-}$ VSMCs. However, the LMW heparin-treated $CD44^{+/+}$ VSMCs demonstrated a significant decrease in growth capacity when compared with the untreated $CD44^{+/+}$ SMCs ($P < .001$). This inhibitory ability of LMW heparin on VSMCs growth could be partially blocked by anti-CD44 mAb (Fig 5, *B*).

CD44 intensity and time course of LMW heparin effect on CD44 expression on cultured mouse VSMCs. Immuno-identification of CD44 expression was detected by one-color flow cytometry analysis. Compared with the non-LMW heparin-treated VSMCs, LMW heparin-treated VSMCs exhibited significantly higher CD44 intensity on the cellular surface, suggesting that LMW heparin upregulated the CD44 expression on the cultured VSMCs (Fig 6, *A*).

$CD44^{+/+}$ VSMCs were cultured with LMW heparin at 100 μg/mL and CD44 expression was examined daily. We found LMW heparin upregulated CD44 expression in the cultured VSMCs. CD44 expression increased gradually and reached the highest level on the fourth day. The expression trend was evaluated after day 5 and revealed that it is quite stable after 5 days, at a relatively lower level. These data are not included. Day 4 is the peak time of CD44 expression induced by LMW heparin, which suggests that LMW heparin upregulated CD44 expression in the cultured VSMCs in a time-dependent manner (Fig 6, *B*).

DISCUSSION

The underlying mechanism responsible for restenosis after successful angioplasty remains incompletely understood. Previous studies have suggested that LMW heparin could inhibit VSMC proliferation, which is the major pathologic change in occlusive disease.[13,14] CD44 is an important cell-surface specific receptor for hyaluronan.[15] There are very few studies defining the role of CD44 in the injury-induced remodeling lesions and some studies have shown controversial results.[16,17] The present study was designed to investigate whether LMW heparin can inhibit the remodeling of injured femoral arteries in mice and furthermore to elucidate the underlying mechanism.

Our previous studies have shown that LMW heparin inhibited bovine VSMC growth and pulmonary vascular remodeling induced by hypoxia in rodents.[5,6] Vascular stenosis and postangioplasty restenosis are typically VSMC proliferative lesions. It is reasonable to hypothesize that LMW heparin could ameliorate the pathologic vascular remodeling in femoral arteries. Clinical studies did not show any beneficial effects of LMW heparin on human vascular restenosis.[2] We developed the present mouse model of transluminal injury such that it might resemble clinical angioplasty and document that LMW heparin induces a significant reduction of neointima formation. By contrast, warfarin had no effects on the remodeling. These results strongly indicate that LMW heparin's beneficial role in inhibiting the proliferative lesion was not related to its anticoagulant property. Some researchers postulated that heparin inhibited smooth muscle cell growth by suppressing the expression of matrix-degrading enzymes and interstitial collagenase.[18] Other studies have shown that heparin prevented growth factors from binding to their receptors, decreasing oncogene expression at the injured site of the artery wall.[19-21] However, the mechanism by which heparin works as an inhibitor against arterial remodeling and why human clinical studies have shown negative results still remain unknown.

The role that CD44 plays during the process of post-angioplasty remodeling is unclear. Some researchers have reported increased CD44 expression in the neointima, implying that CD44 might be a promoting factor that enhances the remodeling process, while others have reported that CD44 might play a protective role inhibiting neointima formation.[22,23] We found that the targeted deficiency of the CD44 gene significantly enhanced neointimal hyperplasia, suggesting that the CD44 gene has implications in the pathologic remodeling process and might play a protective role.

The remodeling response to injury involves the circulating cells that arise from the bone marrow as well as cells from the local arterial wall.[24] CD44 chimeric data demonstrated that both types of chimeric mice with deficient CD44 signal (WT-KO and KO-WT) presented thicker neointima. Mice deficient in all cells (KO-KO) presented with much thicker remodeling than chimeric mice (WT-KO and KO-WT), and both types of chimeric mice

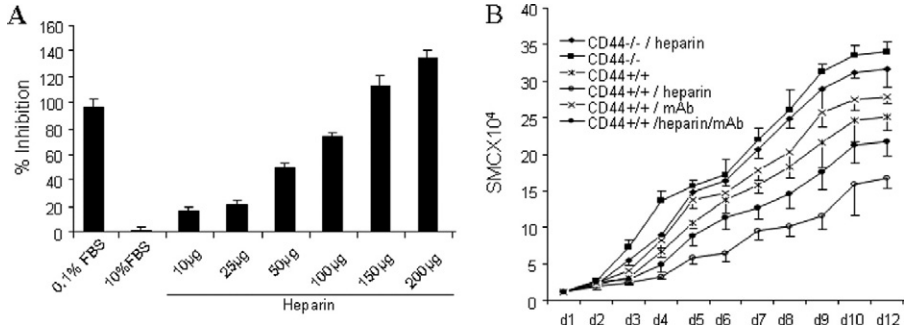

Fig 5. Growth inhibition and growth capacity analysis on the cultured mouse vascular smooth muscle cells (*VSMCs*). Growth inhibition analysis showed low molecular weight (*LMW*) heparin inhibited the cellular growth in a dose-dependent manner. Along with the increasing concentrations, the higher inhibition capacities of LMW heparin on the cultured VSMCs were documented. Conversely, concentrations of LMW heparin at 150 and 200 μg/mL were noted to induce death in a fraction of VSMCs (*the inhibition percentages were both greater than 100%*). The concentration of 100 μg/mL demonstrated the highest inhibition on VSMC growth without causing cells to die (**A**). Growth capacity analysis indicated that LMW heparin significantly inhibited the proliferation of the cultured CD44 +/+ VSMCs and this effect could be partially blocked by the anti-CD44 mAb at a dose of 6 μg/mL ($P < .001$). LMW heparin had no significant effect on the growth capacity of cultured CD44$^{-/-}$ VSMCs ($P > .05$) (**B**).

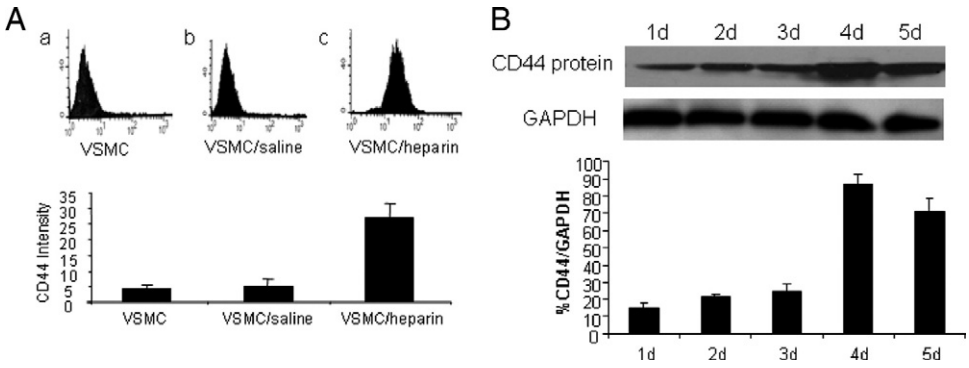

Fig 6. CD44 intensity and time course study on CD44 expression on the cultured mouse VSMCs incubated with low molecular weight (*LMW*) heparin. Immuno-identification of CD44 intensity was performed by one-color flow cytometry analysis and indicated that LMW heparin induced markedly higher CD44 expression on the cultured mouse vascular smooth muscle cells (*VSMCs*). (**A:** *flow cytometry analysis of the CD44 expression on the cultured VSMCs, a, b, and c represent untreated, saline-treated and LMW heparin-treated VSMCs, respectively. Untreated VSMCs vs LMW heparin-treated VSMCs, $P < .005$; untreated VSMCs vs saline-treated VSMCs, $P > .05$*). Time course study on the effects of LMW heparin at a concentration of 100 μg/mL on the expression of CD44 revealed that LMW heparin increased CD44 expression in a time-dependent manner, which reached the highest level on the fourth incubating day (**B:** *Western blot detection of CD44 expression*).

showed similar remodeling. These results strongly indicated that CD44 signal was involved in the process of neointima formation and both the local CD44 signal in the vessel wall and circulating CD44 signal contributed equally to neointima formation in this model.

CD44 is a transmembrane glycoprotein and works as a principal cell receptor for hyaluronic acid, interacting with many extracellular matrix molecules and intracellular signaling molecules.[25] Kothapalli revealed that CD44 possessed an antiproliferative function mediated by differential activation of ERK and Rac genes.[26] Various mechanisms have been proposed, but the biological behavior of CD44 is multivariant, controversial, and still unclear.

Interestingly, we documented that LMW heparin significantly upregulated CD44 expression in neointima. Furthermore, LMW heparin did not reduce the remodeling in injured CD44$^{-/-}$ mice. Additionally, immunoidentification of CD44 expression demonstrated that LMW heparin induced higher CD44 intensity on the cultured mouse VSMCs. These results suggested that LMW heparin might inhibit the remodeling lesion via the CD44 pathway. Meanwhile, LMW heparin reduced VSMCs proliferative capacity, and this action could be partially blocked by anti-CD44 mAb. The in vitro findings were consistent with what we found in the in vivo studies. Taken together, we postulated that LMW hep-

arin inhibited injury-induced femoral artery remodeling in the mouse, at least partially, via upregulating CD44 expression. Kaya reported that heparin might bind to EGF and its receptor and complex with CD44, influencing CD44 expression.[27] However, how LMW heparin upregulates the expression of CD44 is still poorly understood.

The outcomes of these studies have potential clinical implications in treating stenotic vascular disease. VSMC proliferation is the major cause of vascular restenosis. A critical player in regulating VSMC proliferation is CD44 and may represent a target for clinical therapeutic intervention. The finding that heparin upregulates CD44 expression on SMCs suggests that it could be a theoretically practical tool in the upregulation of CD44 expression on VSMCs. In addition, recent technological advances permit the structural modification of heparin to achieve variants of heparin with stronger antiproliferative property and less anticoagulant activity. Although the present research has clinical significance, its use is still a long way from being applicable to actual clinical practice. More work should be performed to elucidate the underlying mechanisms by which heparin prohibits VSMC proliferation, as well as the creation, testing, and selecting of modified heparin variants, before clinical practice may begin.

Collectively, our data suggested that LMW heparin induced higher CD44 expression in VSMCs and that CD44 might play a previously unrecognized role in preventing neointima formation in mice. However, the current study has certain limitations, such as how LMW heparin upregulates CD44 expression and whether LMW heparin affects CD44 expression directly or indirectly. The current study also did not address the role of other genes or factors that might be implicated in the interaction of LMW heparin, CD44 and remodeling, or if these results can be extrapolated to human studies and what the real clinical benefit might be. More studies are still needed to clarify the underlying mechanism of vascular occlusive diseases (Supplementary Figures 1-5, online only).

AUTHOR CONTRIBUTIONS

Conception and design: GZ, RS, HZ, BJ, SK, CH
Analysis and interpretation: GZ, RS, SK
Data collection: GZ, HZ, BJ, SK
Writing the article: GZ, RS
Critical revision of the article: GZ, RS, HZ, BJ, SK, CH
Final approval of the article: GZ, RS, HZ, BJ, SK, CH
Statistical analysis: GZ, RS, SK
Obtained funding: CH
Overall responsibility: GZ, RS, CH

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Additional material for this article may be found online at www.jvascsurg.org.

Appendix, online only

How to calculate the inhibition rate of heparin on cultured VSMCs

Average count of 10% FBS = average of raw counts of 5 wells of VSMCs cultured in 10% FBS

Average count of 0.1% FBS = average of raw counts of 5 wells of VSMCs cultured in 0.1% FBS

Net average of 10% FBS = average count of 10% FBS − Average count of 0.1% FBS

Note:

Inhibition of 10% FBS is theoretically regarded as 0%

Inhibition of 0.1% FBS is theoretically regarded as 100%

Inhibition >100% means a fraction of cultured cells are induced to die

$$\%\ \text{Inhibition} = 1 - \frac{\text{Raw count of samples} - \text{Average count of } 0.1\%\ \text{FBS}}{\text{Net average of } 10\%\ \text{FBS}} \times 100\%$$

Methods and materials

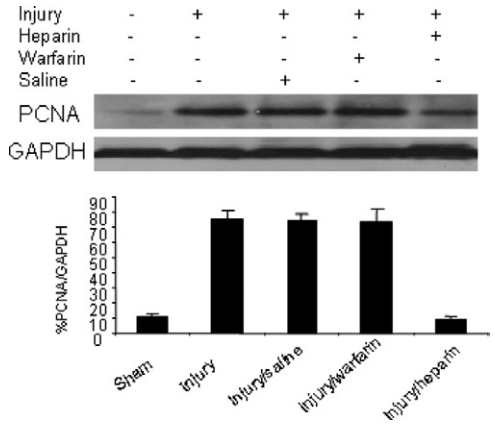
Elastin van Gieson staining. Cross-section slides were deparaffinized and hydrated in distilled water and then underwent Elastin van Gieson staining. These sections were first stained in Verhoff's solution for 30 minutes and then rinsed in tap water for 5 minutes. Sections were subsequently incubated in 2% aqueous ferric chloride solution for 3 minutes and rinsed in tap water for 2 minutes. Next, the sections were treated in 5% sodium thiosulfate for 2 minutes and rinsed in tap water for 5 minutes. Finally, the sections were counterstained in van Gieson's solution for 5 minutes and then dehydrated and covered for morphometric analysis.

Immunohistochemistry assessment. Following the manufacturer's protocol, we used labeled-strepavidin-biotin (LSAB) immunohistochemical kit (Histostain-plus kit; Zymed Laboratory, Inc, Carlsbad, Calif) to detect CD44 protein (rabbit polyclonal antibody; Abcam Inc, Cambridge, Mass) in the femoral arterial neointima. Hematoxylin was used as a counterstain. Negative controls were treated with identical procedures without primary antibody. The results of positive staining were verified by two blinded investigators independently.

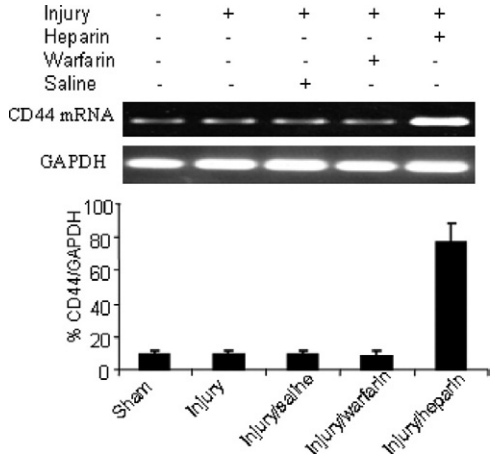
Bone marrow transplantation. After bone marrow single-cell suspensions were prepared in Hank's salt solution under aseptic conditions, male mice that received lethal irradiation (10 Gy in one dose at a rate of 2 Gy/min, MBR-1520r Hitachi irradiator; Hitachi, Tokyo, Japan) were injected with 5×10^6 bone marrow cells via tail vein on the second day following irradiation. Reconstitution of the mice with donor bone marrow was confirmed by detection of CD44 expression by flow cytometry in splenocytes isolated from recipient mice.

Flow cytometry detection of CD44 expression on cultured VSMCs. Briefly, cells suspended in 100 μL of PBS solution were incubated with FITC-conjugated CD44 mAb (FITC rat anti-mouse; BD Pharmingen, San Diego, Calif) for 15 minutes at room temperature. Cells were then washed with PBS and analyzed with a FACScan (Becton Dickinson and Co, Mountain View, Calif). Rat FITC-conjugated IgG2a (BD Pharmingen) was used as a negative control. The experiment was repeated three times.

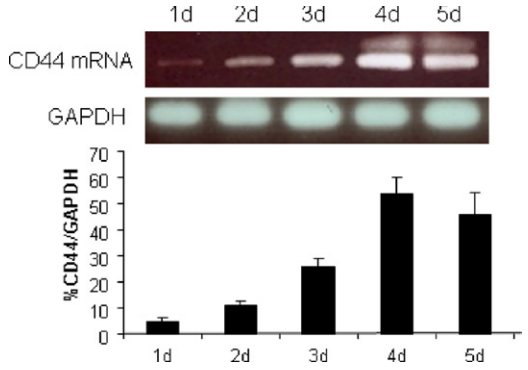
RT-PCR. Total RNA was extracted from femoral arteries (five mice per group) or cultured mouse VSMCs: 1.5 μg total RNA was used to perform RT-PCR to measure the CD44 mRNA level with one-step RT-PCR kit (SuperScript One-step RT-PCR Platinum Tag HiFi Kit; Invitrogen, Carlsbad, Calif). The primer pairs for CD44 were (5′GGCGACTAGATCCCTCCGTT3′; 5′ACCCAGAGGCATACCAGCTG3′) and the housekeeping gene GAPDH was (5′AAGGTCGGAGTCAACGGATT3′; 5′CTGGAAGATGGTGATGGGAT3′) and were purchased from Sigma Genosys (St. Louis, Mo).



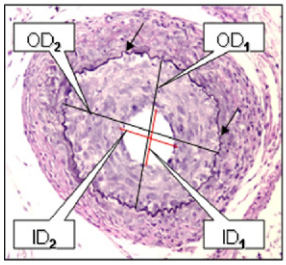
Supplementary Fig 1, online only. Low molecular weight (*LMW*) heparin decreased proliferating cell nuclear antigen (*PCNA*) expression in the injured femoral arteries in CD44 +/+ mice. LMW heparin reduced proliferating cell nuclear antigen (*PCNA*) expression in injured femoral arteries, whereas warfarin- or saline-treated femoral arteries did not show any changes in PCNA expression, indicating an antiproliferative role of LMW heparin in the process of vascular remodeling, and this property of heparin was not due to its anticoagulant activity (*Western blot detection of CD44 expression*). Each group, n = 5. *GADPH*, Glyceraldehyde-3-phosphate dehydrogenase.



Supplementary Fig 2, online only. Low molecular weight (*LMW*) heparin upregulated CD44 mRNA expression in the injured CD44 +/+ femoral arteries. The injured $CD44^{+/+}$ femoral arteries did not show a significant change of CD44 mRNA expression compared with that of the uninjured $CD44^{+/+}$ artery. When treated with LMW heparin, the injured femoral arteries expressed much higher CD44 mRNA (*RT-PCR detection of CD44 mRNA expression*) in the fourth week after the injury, whereas warfarin or saline did not affect the expression of CD44 mRNA. Each group, $n = 5$.



Supplementary Fig 3, online only. Time course study on CD44 mRNA expression on the cultured mouse VSMCs incubated with low molecular weight (*LMW*) heparin. Time course study on the effects of LMW heparin at a concentration of 100 μg/mL on the expression of CD44 mRNA revealed that LMW heparin increased CD44 expression in a time-dependent manner. mRNA expression reached the highest level on the fourth incubating day (*RT-PCR detection of CD44 mRNA expression*). *GADPH*, Glyceraldehyde-3-phosphate dehydrogenase.



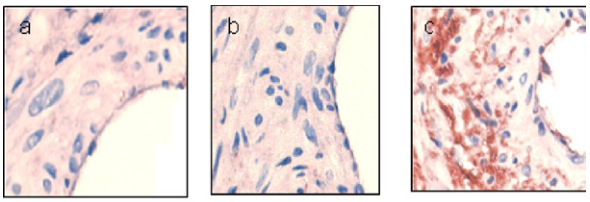
$$mOD = \frac{OD_1 + OD_2}{2}$$

$$mWT = \frac{OD_1 - ID_1 + OD_2 - ID_2}{2}$$

$$WT\% = \frac{mWT}{mOD} \times 100\%$$

mOD: Mean Outer Diameter; OD: Outer Diameter; ID: Inner Diameter;
mWT: Mean Wall Thickness;
WT%: Wall Thickness Percentage.

Supplementary Fig 4, online only. How to measure and calculate the wall thickness percentage (WT%). Based on the image analysis software ImageJ v. 1.33u (National Institutes of Health, Bethesda, Md), we measured the OD_1, OD_2, ID_1, and ID_2, and then calculated mOD, mWT. Finally, we calculated the WT% (*injury-induced remodeled mouse femoral artery, Elastin van Gieson staining, arrows indicate internal elastic lamina*).



Supplementary Fig 5, online only. Low molecular weight (*LMW*) heparin upregulated CD44 protein expression in the injured CD44 +/+ femoral arteries. In $CD44^{+/+}$ mice, the injured arteries did not show a significant change of CD44 expression compared with the uninjured $CD44^{+/+}$ mice. When treated with LMW heparin, the injured femoral arteries expressed much higher CD44 protein (*IHC detection of CD44 protein, a, b, and c represent sham, injured, and injured femoral artery treated with LMW heparin, respectively*).