Vascular Apolipoprotein E Expression and Recruitment from Circulation to Modulate Smooth Muscle Cell Response to Endothelial Denudation

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Apolipoprotein E (apoE) has been shown previously to have anti-proliferative and anti-migratory effects on smooth muscle cells in culture. In addition, overexpression of the apoE gene also reduces neointimal hyperplasia in mice after endothelial denudation. In this investigation, immunohistochemical techniques were used to demonstrate that apoE was present in the medial smooth muscle layers of the carotid artery between 1 and 28 days after endothelial cell denudation. Analysis of transgenic mice overexpressing human apoE in the liver revealed that apoE was recruited from the circulation to the injured site of the vessel wall. *In situ* hybridization using a mouse-specific apoE mRNA probe confirmed that apoE was also synthesized in the carotid artery after endothelial denudation. Interestingly, apoE accumulation in apoE transgenic mice followed a layer-specific pattern, and was inversely associated with smooth muscle α -actin expression. The vascular accumulation of apoE after endothelial denudation, and its association with α -actin-depleted smooth muscle cells, suggest that apoE inhibition of injury-induced neointimal hyperplasia is not due to the inhibition of injury-induced smooth muscle cell de-differentiation, but is likely a direct effect of apoE on smooth muscle cell migration and proliferation. (*Am J Pathol* 2004, 164:2109–2116)

Neointimal hyperplasia of vascular smooth muscle cells (VSMC) is a key pathological process in disease states which involve injurious insults to the vascular wall.¹ Normally quiescent and contractile, VSMC exposed to mechanical and/or chemical stress are activated and begin to proliferate and migrate from the media to the intima, inciting vascular stenosis.^{2–4} The modulation of VSMC phenotype has been implicated as the underlying mechanism for the transformation of inactive and differentiated VSMC into activated cells. The de-differentiated cells lose the typical characteristics of VSMC, become active in extracellular matrix synthesis, and proliferate and migrate in response to growth factor and cytokine stimulation. The

activated VSMC remain metabolically active until the phenotype change is reversed.⁵

One protein that has been shown to modulate vascular response to injury is apolipoprotein (apo) E. This protein is well-known for its role in lipid metabolism, particularly as associated with very low density lipoprotein (VLDL) and some high density lipoprotein (HDL) particles.⁶ ApoE has also been shown to have several anti-atherogenic effects, both through its role in lipid metabolism, as well as through anti-oxidant properties.^{7,8} However, there have been significant findings recently that point toward the role of apoE in the inhibition of smooth muscle cell proliferation and migration.^{9–12} In mouse models, apoE has been shown to modulate the vascular response to injury *in vivo*.^{13,14} Mice in which the apoE gene has been knocked out show an exacerbated response to endothelial denudation compared to wild-type. In contrast, transgenic mice with liver overexpression of the human apoE gene show a decreased response to vascular injury in the form of neointimal hyperplasia.¹³ Ishigami et al^{9,10} showed that apoE inhibits growth-factor-induced smooth muscle cell migration and proliferation *in vitro*, suggesting that apoE inhibition of arterial response to injury may be a direct result of its modulation of smooth muscle cell response to stimulant.

Despite demonstrating the importance of apoE level in serum suppressing neointimal hyperplasia after endothelial denudation, a direct interaction between the circulating apoE and cells in the arterial wall has not been documented previously. It remains possible that apoE in circulation acts indirectly, for example through paracrine mechanisms, in modulation of arterial response to injury. Additionally, apoE has also been shown to be synthesized by quiescent smooth muscle cells in culture.^{15,16} Whether this vascular apoE also contributes to the modulation of arterial response to injury is unclear. Moreover, apoE expression pattern in the vessel wall after endothelial denudation has not been explored. This study endeavors to determine whether a direct interaction between apoE and the denuded tissue of the carotid artery exists, and additionally, whether the source of apoE is via

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local expression or recruitment from the circulation. Moreover, the relationship between apoE-dependent inhibition of VSMC neointimal hyperplasia and phenotypic modulation of VSMC was also examined.

Materials and Methods

Animals

Transgenic mice with liver-specific human apoE gene expression were generously provided by Dr. John Taylor (Gladstone Institute, San Francisco, CA) and were backcrossed in our institution to the FVB/N background (>99% genetic homogeneity). Progeny were evaluated for the expression of the human apoE transgene by determining human apoE level in serum samples. Typically, the serum concentration of human apoE in these transgenic mice averaged 30 ± 5 mg/dL, which was 7 to 15 times higher than the level of mouse apoE in wild-type mice. Both wild-type and transgenic progeny were used in this study, with FVB/N wild-type mice serving as non-transgenic sibling controls. Wild-type FVB/N and C57BL/6 mice were obtained from The Jackson Laboratory (San Francisco, CA). The animals were maintained on a 12-hour light/12-hour dark cycle and were fed a normal mouse chow diet (Harlan Teklad, Madison, WI). Food and water were available *ad libitum*. All animals used for experimentation were male, between 6 to 8 weeks of age, and weighed between 25 to 30 grams. All animal experimentation protocols were performed under the guidelines of animal welfare prescribed by the University of Cincinnati, in accordance with National Institutes of Health guidelines.

Human ApoE Assay

Enzyme-linked immunosorbent assay (ELISA) was used to determine the presence of human apoE in the progeny of transgenic and wild-type FVB/N mice. A 96-well microtiter plate was incubated overnight with 100 μ l of a 2 mg/ml solution of mouse anti-human apoE monoclonal antibody 1D7 (Ottawa Heart Institute Research Corporation, Ottawa, Ontario, Canada). Nonspecific sites were then blocked with phosphate-buffered saline (PBS) containing 5 mg/ml Tween-20 and 5 mg/ml bovine serum albumin. Mouse serum samples were diluted 1:1000 in PBS and added to each well. Plates were then incubated for 1 hour at 37°C, then washed with a solution of PBS and 1% Tween-20. A 1:500 dilution of rabbit anti-human apoE polyclonal antibody (DAKO, Carpinteria, CA) in PBS was then added to the plate, and allowed to incubate for 1 hour at 37°C. The plates were washed and then incubated for an additional hour at 37°C with a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG in PBS (Sigma Chemical Co., St. Louis, MO). Immunoreactivity was determined by addition of ALP-10 substrate (Sigma) and measuring absorbance at 405 nm. Purified human apoE isolated according to Rall et al¹⁷ was used as the standard.

Carotid Artery Injury

Mechanically induced endothelial denudation was performed by modification of the Lindner¹⁸ method as described^{13,19}. In this procedure, the denudation probe was constructed by coating a 3-0 nylon suture with an epon-resin plastic mixture, similar to that used to fix samples for electron microscopy. Beads of resin were formed on the suture, whose diameter was reduced to slightly larger than the diameter of the carotid artery (0.45 mm). The animals were anesthetized by intraperitoneal injection with a solution composed of ketamine (80 mg/kg body weight; Fort Dodge Laboratories, Inc., Fort Dodge, IA) and xylazine (16 mg/kg; The Butler Co., Columbus, OH) diluted in 0.9% NaCl. The mice were then immobilized and the fur covering the neck from sternum to chin was removed with lotion hair remover (Nair; Carter-Wallace, Inc., New York, NY). Surgery was performed under a dissection microscope (Leica GZ6; Leica, Buffalo, NY). The entire length of the left carotid artery was exposed and the artery was ligated immediately proximal from the point of bifurcation with a 7-0 silk suture (Ethicon, Inc., Somerville, NJ). Another 7-0 suture was placed around the common carotid artery immediately distal from the branch point of the external carotid. A transverse arteriotomy was made between the 7-0 sutures and the resin probe was inserted and advanced toward the aorta arch and withdrawn five times. The probe was removed and the proximal 7-0 suture was ligated. Once restoration of blood flow through the carotid branch points was confirmed, the incision was closed with a 5-0 sterile surgical gut (Ethicon, Inc.). The entire procedure was performed within 20 minutes. Animals were allowed to recover in a 37°C heat box. The identical surgical procedure was applied to each animal to assure reproducibility of the results.

Tissue Preparation

Animals were anesthetized and perfused with 0.9% NaCl by placement of a 22-gauge butterfly angiocatheter in the left ventricle. The mice were subsequently perfusion-fixed *in situ* by infusion with 4% phosphate-buffered paraformaldehyde and 0.05% DEPC-treated water (pH 7.0) for 20 minutes at a constant pressure of 100 mm Hg. Both denuded and control arteries were dissected from each mouse and fixed in paraformaldehyde-PBS solution for an additional 24 hours. Tissues were saturated overnight at 4°C in 30% sucrose in PBS buffer, and then embedded in OCT (Sakura Finetek U.S.A. Inc., Torrance, CA) and flash frozen in liquid nitrogen. Cross sections of the arteries were cut at a thickness of 5 μ m and placed on TESPA-treated slides, then stored at -80°C until use.

Immunohistochemistry

For all staining, sections were allowed to assume room temperature, dehydrate, and then fixed for 10 minutes in acetone at -20°C. Slides were then washed in PBS for 5 minutes, and endogenous peroxidase activities were

blocked by incubating for 30 minutes with 0.3% hydrogen peroxide and 0.3% normal horse serum (Vector Laboratories, Inc., Burlingame, CA) in PBS containing Triton X-100 (Sigma Chemical Co.). The slides were then washed three times in PBS containing Triton X-100. Non-specific binding sites were blocked by incubation for 30 minutes with PBS containing 1.5% normal serum. All sections were incubated overnight at 4°C with primary antibodies to identify the antigen of choice. Mouse apoE was detected using a polyclonal goat anti-mouse apoE antibody (Clone M-20, Santa Cruz Biotechnology, Santa Cruz, CA). Human apoE was detected using a monoclonal mouse anti-human apoE antibody (Clone A1.4, Santa Cruz Biotechnology). The identification of α -actin-positive smooth muscle cells was performed using a monoclonal mouse anti-smooth muscle α -actin antibody (Clone 1A4; Sigma Chemical Co). All primary antibodies were administered at a 1:1000 dilution in Antibody Diluent (Zymed, San Francisco, CA). A 1-hour pre-incubation step with Mouse IgG Blocking reagent (Vector Laboratories, Inc.) was performed before antigen detection with mouse anti-human apoE and the mouse anti-smooth muscle α -actin antibodies. The slides were washed three times for 15 minutes each with PBS containing Triton X-100 and then incubated for 1 hour at 23°C with either 0.5% biotinylated anti-mouse IgG (Vector Laboratories, Inc.) for anti-human apoE and anti-smooth muscle cell α -actin detection, or 0.5% biotinylated anti-goat IgG (Vector Laboratories, Inc.) for anti-mouse apoE detection in the same solution containing 1.5% normal serum. Slides were then washed as described above, and then incubated with the avidin-peroxidase complex reagent (Peroxidase Vectastain Elite ABC kit, Vector Laboratories, Inc.) for 1 hour at 23°C. The reaction was visualized with 3-amino-9-ethylcarbazole (Vector Laboratories, Inc.) for anti-mouse apoE and anti-human apoE, and Vector NovaRED (Vector Laboratories, Inc.) was used to visualize anti- α -actin reaction. All sections were counterstained with Hematoxylin QS (Vector Laboratories, Inc.). Sections were mounted using aqueous GVA mounting medium (Zymed) for anti-mouse apoE and anti-human apoE sections, and VectaMount permanent mounting medium (Vector Laboratories, Inc.) for anti- α -actin sections.

In Situ Hybridization

In situ hybridization was carried out on parallel tissue sections prepared as described above. Sections were hybridized to a mouse-specific apoE cDNA probe (Clone ID 733015) obtained from the American Type Culture Collection (Manassas, VA). The mouse apoE cDNA in a pBluescript SK vector was transfected into *E. coli*, grown in a 1-L culture, and the plasmid DNA was retrieved through phenol-chloroform extraction. The apoE cDNA probe was excised from the vector by *Eco*RI and *Xho*I digestion and then labeled with biotin using Biotin RNA Labeling mix (Roche Diagnostics Corporation, Indianapolis, IN). Probes were then purified by passage through a G-50 Sephadex column specific for biotinylated RNA (Roche Diagnostics Corporation). Tissues were hybrid-

Figure 1. Representative immunohistochemical staining for apoE in uninjured (**a** and **b**) and injured (**c** and **d**) carotid arteries of C57BL/6 wild-type mice (**a** and **c**) and *apoE* knockout mice (**b** and **d**) 5 days after endothelial denudation. Positive immunoreactivity with anti-mouse apoE antibodies was denoted by the presence of red precipitates in the micrographs. The images were captured with a $\times 40$ objective.

ized overnight at 42°C on an Omnislide Hybridizer (Thermo Hybaid, Middlesex, UK). Positive signal was detected using a commercially available BIO-AP REMBRANDT Universal ISH Detection kit (Zymed). The provided protocol used an anti-biotin antibody conjugated to peroxidase, which was then visualized with 3-amino-9-ethylcarbazole, and counterstained with Hematoxylin QS (Vector Laboratories, Inc.).

Immunofluorescent co-localization of apoE and apoA-I epitopes was performed using the anti-human apoE antibody clone A1.4 described above and a polyclonal goat anti-mouse apoA-I antibody (ab7614, Abcam Inc., Cambridge, MA). Tissues were nonspecifically blocked as described above and then incubated overnight with the primary antibodies. Anti-apoE antibody was detected with Alexa Fluor 488 donkey anti-mouse IgG (A-21202, Molecular Probes) and anti-apoA-I antibody was detected with Alexa Fluor 594 donkey anti-goat IgG (A-11058, Molecular Probes). All tissues were counterstained with the nonspecific nuclear counterstain DAPI (D-21490, Molecular Probes).

Results

The direct interaction of apoE with quiescent and activated vessel walls was explored after endothelial denudation of the left carotid arteries of C57BL/6 mice. After a recovery period of 5 days, mice were sacrificed and their carotid arteries were isolated for tissue sectioning. Fixed sections from both denuded and control carotid arteries were immunohistochemically probed with an anti-mouse apoE antibody. Although occasional nonspecific red precipitate was observed in the adventitia in both control and denuded arteries, red precipitate correlating to positive apoE immunoreactivity was observed in high quantities in the medial layers of denuded vessels. Control uninjured vessels showed minimal to no apoE accumulation in the media (Figure 1). Specificity of the anti-mouse apoE antibody was confirmed by the lack of immunoreactivity in the injured vessel of *apoE* knockout mice (Figure 1).

The physiological origin of the apoE in the injured arteries was investigated by taking advantage of the

Figure 2. Representative immunohistochemical staining for apoE in injured (a and c) and uninjured (b and d) carotid arteries of FVB/N wild-type mice 1 day (a and b) and 28 days (c and d) after endothelial denudation. Positive immunoreactivity with anti-mouse apoE antibodies was denoted by the presence of red precipitates observed in the micrographs. The images were captured with $\times 40$ objective.

availability of liver-specific human *apoE* transgenic mice as well as antibodies that distinguish human and mouse apoE. Because the liver-specific human *apoE* transgenic mice were backcrossed to FVB/N background, wild-type FVB/N mice were used as the control for comparison. Similar to results observed with C57BL/6 mice, mouse apoE was found to be present in the medial layers of injured arteries but not in the uninjured control arteries of wild-type FVB/N mice. A more detailed time course study revealed the presence of mouse apoE in the injured carotid arteries of FVB/N mice as early as 24 hours after endothelial denudation (Figure 2). Positive apoE immunoreactivity remained present in the medial layer of the injured arteries in FVB/N mice even after 28 days (Figure 2). The possibility that the apoE present in the denuded arteries was originated from plasma circulation was then explored by immunohistochemical examination of carotid arteries from the liver-specific human *apoE* transgenic mice. Using monoclonal mouse anti-human apoE antibodies as the probe, the data showed positive immunoreactivity indicative of the presence of human apoE in the injured but not the uninjured carotid arteries of the human apoE transgenic mice at both the 1- and 28-day time points (Figure 3). Specificity of the antibodies for human *apoE* was confirmed based on the lack of positive signal in the wild-type mice (data not shown). The use of the polyclonal goat anti-mouse apoE antibodies also revealed positive reactivity in the medial layers of the injured carotid arteries in these animals (Figure 3). Neither specific human nor mouse apoE immunoreactivity was observed in the control uninjured arteries of the transgenic mice (Figure 3). Note that despite the extensive vascular perfusion that was consistently used throughout all experiments, we also observed the presence of circulating blood cells in the lumen of the injured arteries of *apoE* transgenic mice 24 hours after endothelial denudation. This may be due to incomplete perfusion or to a specific recruitment and retention of circulating blood cells in the injured vessels of *apoE* transgenic mice. The latter possibility is consistent with previous observation that circulating lymphocytes are transiently recruited to the injured vessel wall after endothelial denudation and that T and B lymphocytes serve a protective role against

Figure 3. Representative immunohistochemical staining for apoE in injured (a, c, e, and g) and uninjured (b, d, f, and h) carotid arteries of liver-specific human *apoE* transgenic mice with antibodies against either human apoE (a–d) or mouse apoE (e–h) at 1 day (a, b, e, and f) and 28 days (c, d, g and h) after endothelial denudation. Positive immunoreactivity with the apoE antibodies was denoted by the presence of red precipitates observed in the micrographs. The images were captured with $\times 40$ objective.

injured-induced neointimal hyperplasia.¹⁴ Whether the resistance of *apoE* transgenic mice to injury-induced neointimal hyperplasia¹³ is related to the increased recruitment and retention of lymphocytes at the site of injury remains to be determined. These circulating cells also displayed apparent positive immunostaining with anti-apoE antibodies. However, since human apoE is not expressed in circulating cells in the liver-specific human apoE transgenic mice, the signal observed in the circulating cells was likely nonspecific due to the excessive amount of peroxidase found in these cells.

The presence of the liver-derived human apoE in the injured arteries of the transgenic mice suggested that apoE in circulation was recruited to the denuded artery after endothelial denudation. In view of the fact that apoE circulates in plasma primarily associated with HDL particles, additional experiments were performed to determine whether other HDL-associated apolipoproteins, such as apoA-I, may also be present in the vessel wall after endothelial denudation. Immunofluorescence analysis with anti-apoE and anti-apoA-I antibodies revealed the presence of both of these apolipoproteins in the vessel wall of FVB/N wild-type mice after endothelial denudation (Figure 4). The merged image of the immunofluorescence data showed co-localization of both of these apolipoproteins, thus suggesting that apoE may be recruited from circulation in association with HDL. Importantly, apoA-I was also found to be present in the injured arteries of apoE knockout mice (Figure 4). Nevertheless, rampant neointimal hyperplasia was consistently observed in the *apoE* knockout mice.^{13,14} Thus, the infiltra-

Figure 4. Immunofluorescent detection of apoE and apoA-I in injured carotid arteries of FVB/N wild-type mice (**a–c**) and *apoE* knockout mice (**e–g**). The presence of apoE in the vessel wall was detected by anti-apoE antibodies (**a**, **e**) with green signals indicative of positive reaction. The presence of apoA-I (**b** and **f**) was detected by anti-apoA-I antibodies with red fluorescence indicative of positive reaction. **c** and **g**: The merged fluorescent images of **a** and **b** and **e** and **f**, respectively, with the co-localization of apoE and apoA-I immunoreactivity showing a yellowish fluorescence. **d** and **h**: The negative controls showing the absence of apoA-I in the uninjured carotid arteries of FVB/N and *apoE* knockout mice, respectively.

tion of HDL to the vessel wall after endothelial denudation is not sufficient, and the presence of apoE is required, for protection against injury-induced neointimal hyperplasia.

Previous studies have documented the ability of arterial smooth muscle cells to synthesize apoE *in vitro*. Accordingly, additional experiments were performed to determine whether the apoE present in the injured arteries may also be derived locally by the arterial smooth muscle cells. In these experiments, tissues from wild-type and human *apoE* transgenic FVB/N mice were analyzed by *in situ* hybridization with a mouse-specific apoE mRNA probe. Results clearly showed the presence of mouse apoE mRNA in the denuded arteries of both wild-type and human *apoE* transgenic mice 1 day after endothelial denudation (Figure 5). Interestingly, mouse apoE mRNA was also present in the contralateral uninjured arteries 1 day after the experimental procedure (Figure 5). Moreover, the data also revealed that apoE gene transcriptional activity in both the injured and the contralateral uninjured arteries, as monitored by the presence of apoE mRNA, had ceased by 14 days after endothelial denudation. Taken together, these results indicated apoE was expressed in both the denuded and the undenuded arteries during the early phase of vascular response to injury. In contrast, apoE was not expressed by quiescent smooth muscle cells in the vessel wall at the completion of endothelial repair. The apoE synthesized in the uninjured arteries during the early phase was not retained within the arterial wall as no apoE immunoreactivity was detectable in the uninjured arteries of either FVB/N wild-type or *apoE* transgenic mice. In contrast, apoE synthesized or recruited to the injured arteries during the initial period was retained even after endothelial repair was completed (compare Figures 3 and 5).

During the course of this investigation, we observed the presence of apoE in a layer-specific manner in the vessel wall throughout the time period of this study (Fig-

ure 6). Specifically, the most intense apoE immunoreactivity was observed in the abluminal medial layer, especially during days 7 to 14 after endothelial denudation. This observation prompted us to investigate a possible relationship between apoE accumulation and smooth muscle cell activation after endothelial denudation. An anti- α -smooth muscle actin antibody was used to analyze parallel sections for expression of α -smooth muscle actin. These sections showed that the areas of the media with prominent apoE accumulation were also areas where dramatic decrease in α -smooth muscle actin expression was observed (Figure 7). This inverse relationship indi-

Figure 5. *In situ* hybridization of mouse apoE mRNA in carotid arteries of FVB/N wild-type and *apoE* transgenic mice. The injured carotid arteries (**a**, **c**, and **e**) and the uninjured contralateral arteries (**b**, **d**, and **f**) were obtained from wild-type (**c**, **d**, and **e**) and *apoE* transgenic (**a**, **b**, and **f**) mice at day-1 (**a–d**) or day-14 (**e** and **f**) after endothelial denudation. The tissues were incubated with antisense apoE mRNA probe for the detection of apoE mRNA. Red staining in the micrographs represents positive reaction with the antisense apoE probe.

Figure 6. Recruitment and accumulation of mouse and human apoE in the injured carotid arteries of liver-specific human *apoE* transgenic mice. Endothelial denudation was performed in the carotid arteries of *apoE* transgenic mice. The injured arteries were harvested at day-5 (**a** and **b**), day-7 (**c** and **d**), day-10 (**e** and **f**), day-14 (**g** and **h**), and day-28 (**i** and **j**) after endothelial denudation. Tissues were immunostained with antibodies against either mouse apoE (**a**, **c**, **e**, **g**, and **i**) or human apoE (**b**, **d**, **f**, **h**, and **j**). Positive immunoreactivity was indicated by the presence of red staining. The images were captured with a $\times 40$ objective.

cated that apoE in the vessel wall did not inhibit smooth muscle cell phenotype conversion after endothelial denudation, suggesting that its inhibition of neointimal hyperplasia is mediated through direct inhibition of smooth muscle cell migration and proliferation.

Discussion

The current study took advantage of inbred mouse strains differing in their resistance or susceptibility to injury-induced neointimal hyperplasia to explore the potential direct involvement of apoE in cellular homeostasis in the vessel wall. The C57BL/6J strain has been demonstrated to be resistant to the formation of neointimal hyperplasia after endothelial denudation, whereas the FVB/N strain has been shown to be susceptible.²⁰ The resistance or susceptibility of these two strains was reversed, however, with the deletion or addition of the apoE gene.¹³ The deletion of the apoE gene in C57BL/6J mice caused a dramatic increase in neointimal hyperplasia after arterial injury, whereas overexpression of apoE in FVB/N mice resulted in resistance to neointimal formation.¹³ It is important to note, in the context of experiments

Figure 7. Inverse relationship between smooth muscle α -actin and apoE immunostaining in parallel carotid sections in the human *apoE* transgenic mice. Denuded carotid arteries were obtained at day-1 (**a–d**) and day-10 (**e–h**) after injury and used for immunohistochemical analysis with antibodies against either smooth muscle α -actin (**a**, **b**, **e**, and **f**) or human apoE (**c**, **d**, **g**, and **h**). Black arrowheads in the micrographs identified regions of parallel sections in which increased apoE accumulation and decreased smooth muscle α -actin expression were observed. The images were captured with a $\times 40$ objective.

in this study, that the transgenic FVB/N mice in these studies overexpressed the human *apoE* transgene specifically in the liver,^{21,22} thereby establishing a correlative relationship between circulating apoE level with resistance to neointimal hyperplasia after arterial injury.

The mechanism by which plasma apoE level influences vascular cell response to injury has not been explored previously. The current study used an immunohistochemical approach to assess the potential of a direct apoE effect at the vessel wall. Results showed specific apoE accumulation in the vessel wall of both wild-type C57BL/6 and FVB/N mice after endothelial denudation. It is important to note that, although both C57BL/6 and FVB/N mice possessed a similar level of apoE circulating in plasma and both strains accumulated apoE in the vessel wall after endothelial denudation, these two strains of mice displayed dramatically different vascular response to injury. These observations suggest that the mechanism(s) dictating the different arterial response to injury in these two mouse strains is (are) independent of the role of apoE in vascular protection. Alternatively, it is also possible that these two mouse strains differ in the extent of vascular apoE synthesis and/or the recruitment and retention of apoE in the vessel wall. Because the anti-mouse apoE antibodies also react with human apoE and liver-specific human *apoE* transgenic mice are available only in FVB/N background, the current study does not allow us to differentiate between these possibilities and to determine the importance of locally derived apoE

versus the importance of apoE recruitment in vascular protection. In studies of diet-induced atherosclerosis in *apoE*-knockout mice, either local synthesis of apoE in the vessel wall,²³ or low level of circulating apoE,²⁴ was shown to be sufficient independently in protecting against foam cell lesions. Our previous study showed that normal circulating level of apoE was not sufficient to protect FVB/N mice from injury-induced neointimal hyperplasia.¹³ Nevertheless, high level of circulating apoE, due to liver-derived transgenic apoE expression, inhibited injury-induced neointimal hyperplasia. Thus, circulating apoE can at least modulate the severity of vascular response to injury in FVB/N mice. Whether increasing local vascular synthesis of apoE can also inhibit injury-induced neointimal hyperplasia remains to be determined. Additionally, whether a low level of circulating apoE can protect C57BL/6 mice against injury-induced neointimal hyperplasia will require additional study examining vascular response to injury in the low-apoE expressing mice.²⁴

The current study also showed that the highest vascular apoE accumulation was observed 5 days after endothelial denudation, but the presence of apoE in the injured arteries was detected as early as 1 day and persisted for at least 28 days even after endothelial repair was complete. The apoE accumulated in the injured arteries was derived from plasma circulation, as demonstrated by the presence of human apoE in the vessel wall of transgenic mice with liver-specific expression of human apoE, as well as from local sources as documented by *in situ* hybridization detection of apoE mRNA. ApoE mRNA was also detected in the uninjured contralateral carotid artery 1 day after endothelial denudation of the other carotid artery. The surgery-induced apoE gene expression in both the injured and uninjured contralateral arteries was no longer detectable after 14 days, suggesting that vascular expression of apoE may be an inflammatory response to the surgical procedure. Interestingly, although the *in situ* hybridization studies revealed the presence of apoE mRNA in both the injured and the uninjured arteries after endothelial denudation, apoE protein was detected only in the injured arteries and not detectable in the uninjured contralateral artery. These observations indicated that apoE synthesized in the vasculature with an intact endothelium is probably secreted into the plasma circulation. In contrast, the denuded arteries were capable of retaining apoE in the local environment where it is readily available to limit neointimal hyperplasia.

Additional examination of apoE accumulation during the time course of vascular response to endothelial denudation revealed an interesting distribution pattern. The apoE protein appeared to accumulate in a layer-specific manner in the media beginning 5 days after endothelial denudation (Figure 6). Previous studies have already demonstrated distinct characteristics of the smooth muscle cells in different layers of the arterial media, which can be correlated to the expression of various molecular markers such as smooth muscle myosin, Ki67, smoothelin, and smooth muscle α -actin.²⁵ The current study showed that both the locally derived apoE (mouse apoE) and apoE recruited from circulation (human apoE in the

transgenic mice) accumulated in the inner-middle layer of arterial media after endothelial denudation. Surprisingly, in the apoE transgenic mice, areas of the vessel wall in which apoE accumulation were most intense were also areas in which smooth muscle α -actin expression was decreased (Figure 6). This pattern persisted through the time course of the study, up through 14 days when the entire medial layer of the vessel was uniformly stained for α -actin expression (data not shown). This pattern of α -actin down-regulation was not observed in control vessels, or the injured and uninjured arteries of wild-type mice (data not shown).

Based on the association of intense apoE accumulation with decreased smooth muscle α -actin expression in injured arteries of the transgenic mice, we can speculate on the mechanism of apoE inhibition of injury-induced neointimal hyperplasia, linking apoE effects to the modulation of smooth muscle cell phenotype in response to arterial injury. It is well documented that arterial smooth muscle cells can be classified into either a quiescent contractile phenotype or an active synthetic phenotype.²⁶ The quiescent phenotype is associated with the steady-state muscle-like nature of smooth muscle cells that exist in arteries without any molecular or physical stimulus. The quiescent smooth muscle cells can be activated to the synthetic phenotype associated by exposure to mitogens and chemokines, resulting in increased rates of cell proliferation, migration, and protein synthesis and secretion. This phenotype conversion from a quiescent state to an active smooth muscle cell is accompanied by transient changes in cytoskeletal proteins, including the decreased expression of smooth muscle α -actin. The observation of increased apoE accumulation in the medial layer of the transgenic mice, along with their decreased neointimal formation after arterial injury, suggests that apoE does not inhibit smooth muscle activation as indicative by phenotype conversion. Rather, apoE inhibits neointimal hyperplasia by preventing the migration of the activated cells from the media to the intima and their subsequent proliferation in the intima. This hypothesis is consistent with our previous studies showing apoE directly inhibits the migration and proliferation of smooth muscle cells *in vitro*.

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