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INCREASED CONNEXIN43 EXPRESSION IN HUMAN SAPHENOUS VEINS IN CULTURE IS ASSOCIATED WITH INTIMAL HYPERPLASIA

THESE

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L'augmentation de l'expression de la Connexine43 est associée à l'hyperplasie intimale dans les veines saphènes humaines en culture

Objectif : L'hyperplasie intimale est un processus de remodelage vasculaire qui apparaît après une lésion vasculaire. Les mécanismes impliqués dans l'hyperplasie intimale sont la prolifération, la dédifférentiation et la migration des cellules musculaires lisses depuis la média vers l'espace sous-intimal. Nous avons émis l'hypothèse que les jonctions communicantes de type gap, qui coordonnent certains processus physiologiques tels que la croissance et la différentiation cellulaire, pouvaient participer au développement de l'hyperplasie intimale.

Méthodes : Des segments de veines saphènes humaines prélevées chirurgicalement lors de pontages, ont été ouverts longitudinalement avec la surface luminale placée vers le haut et maintenus en culture pendant 14 jours. Des fragments veineux ont été préparés pour une évaluation histologique, pour des mesures de l'épaisseur de la néointima, et pour des analyses immunocytochimiques de l'ARN messager ainsi que des protéines.

Résultats : Parmi les 4 connexines (Cxs 37, 40, 43 et 45) qui forment les jonctions communicantes dans les veines, nous avons focalisé notre étude sur l'expression des Cxs 43 et 40; nous avons démontré que la Cx43 est exprimée dans les cellules musculaires lisses et les cellules endothéliales alors que la Cx40 est uniquement présente dans l'endothélium. Après 14 jours en culture, des analyses histomorphométriques ont montré une augmentation significative de l'épaisseur de l'intima démontrant la présence d'hyperplasie intimale. Une analyse temporelle a révélé une augmentation progressive de la Cx43 jusqu'à une augmentation maximale de six à huit fois au niveau de l'ARN messager et des protéines après 14 jours en culture. Au contraire, l'expression de la Cx40 n'était pas modifiée. Des analyses par immunofluorescence ont montré également une augmentation de la Cx43 dans les membranes des cellules musculaires lisses de la média. Le développement de l'hyperplasie intimale *in vitro* est diminué en présence de fluvastatin et cette diminution est associée à une réduction de l'expression de la Cx43.

Conclusions : Ces données démontrent que la Cx43 est augmentée *in vitro* pendant le processus d'hyperplasie intimale et que la fluvastatin prévient cette induction. Ces résultats suggèrent un rôle crucial joué par la communication intercellulaire impliquant la Cx43 dans la veine humaine durant le développement de l'hyperplasie intimale.

Increased connexin43 expression in human saphenous veins in culture is associated with intimal hyperplasia

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Objective: Intimal hyperplasia is a vascular remodelling process that occurs after a vascular injury. The mechanisms involved in intimal hyperplasia are proliferation, dedifferentiation, and migration of medial smooth muscle cells towards the subintimal space. We postulated that gap junctions, which coordinate physiologic processes such as cell growth and differentiation, might participate in the development of intimal hyperplasia. Connexin43 (Cx43) expression levels may be altered in intimal hyperplasia, and we therefore evaluated the regulated expression of Cx43 in human saphenous veins in culture in the presence or not of fluvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase activity.
Methods: Segments of harvested human saphenous veins, obtained at the time of bypass graft, were opened longitudinally with the luminal surface uppermost and maintained in culture for 14 days. Vein fragments were then processed for histologic examination, neointimal thickness measurements, immunocytochemistry, RNA, and proteins analysis.
Results: Of the four connexins (Cx37, 40, 43, and 45), we focused on Cx43 and Cx40, which we found by real-time polymerase chain reaction to be expressed in the saphenous vein because they are the predominant connexins expressed by smooth muscle cells and endothelial cells. After 14 days of culture, histomorphometric analysis showed a significant increase in the intimal thickness as observed during the process of intimal hyperplasia. A time-course analysis revealed a progressive upregulation of Cx43 to reach a maximal increase of sixfold to eightfold at both transcript and protein levels after 14 days in culture. In contrast, the expression of Cx40, abundantly expressed in the endothelial cells, was not altered. Immunofluorescence showed a large increase in Cx43 within smooth muscle cell membranes of the media layer. The development of intimal hyperplasia in vitro was decreased in presence of fluvastatin and was associated with reduced Cx43 expression.
Conclusions: These data show that Cx43 is increased in vitro during the process of intimal hyperplasia and that fluvastatin could prevent this induction, supporting a critical role for Cx43-mediated gap-junctional communication in the human vein during the development of intimal hyperplasia. (J Vasc Surg 2005;41:1043-52.)

Clinical Relevance: Stenosis due to intimal hyperplasia is the most common cause of failure of venous bypass grafts. To better understand the development of intimal hyperplasia, we used an ex vivo organ culture model to study saphenous veins harvested from patients undergoing a lower limb bypass surgery. In this model, the morphologic and functional integrity of the vessel wall is maintained and significant intimal hyperplasia development occurs after 14 days in culture. We have postulated that gap junctions, which coordinate physiologic processes such as cell growth and differentiation, may participate in the development of intimal hyperplasia. Indeed, intimal hyperplasia consists of proliferation and migration of smooth muscle cells into the subendothelial space. Intercellular communication is responsible for the direct transfer of ions and small molecules from one cell to the other through gap-junction channels found at cell-cell appositions. No study to date has evaluated whether gap junctional communication is involved in the process of intimal hyperplasia in humans. This assertion was investigated by using the aforementioned organ culture model of intimal hyperplasia in human saphenous veins, and our data support a critical role for Cx43-mediated gap junctional communication in human vein during the development of intimal hyperplasia.

Stenosis in venous bypass grafts corresponding to intimal hyperplasia is a vascular pathophysiologic feature that may result in occlusion of the vessel lumen.[1,2] Graft wall remodelling is caused by adaptation of the vein to extrinsic factors such as modified shear stress,[3] wall tension,[3] endothelial damage,[4] and platelet activation.[5-7] Intrinsic properties of endothelial cells and medial smooth muscle cells are also involved in intimal hyperplasia development by secreting factors such as platelet-derived growth factor,[5-7] fibroblast growth factor,[8] and insulin-like growth factor.[4] These different factors are implicated in proliferation and migration of medial smooth muscle cells into the subintimal space and are associated with a phenotypic modification of smooth muscle cells. From a quiescent contractile state, the activated smooth muscle cells become prolifera-

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tive and synthetic.[9,10] Thus, smooth muscle cells produce extracellular matrix that composes 80% of the typical IH lesion; smooth muscle cells compose the remaining 20%.

Autologous saphenous vein grafts remain the conduit of choice for aortocoronary and peripheral bypasses, with a patency rate of nearly 60% at 10 years.[11] The limited patency rate of these vascular procedures is due to intimal hyperplasia development, which leads to late graft occlusion.[12] Thus, improvement of the patency rates of these procedures by inhibition of neointimal thickening is a major goal in vascular research. To understand the development of intimal hyperplasia, different organ culture models of human saphenous vein have been proposed.[13-16] We have recently developed an ex vivo setting designed for intimal hyperplasia development that uses native human saphenous vein segments. In this model, the morphologic and functional integrity of the vessel wall is maintained, and significant intimal hyperplasia development occurs after 14 days in culture.[13,14,17-21]

Intercellular communication is responsible for the direct transfer of ions and small molecules from one cell to the other through gap-junction channels found at cell-cell appositions.[22] Molecular cloning studies have shown that gap junctions are formed by members of a family of transmembrane proteins named connexins[22] that are involved in coordinating the electrical and metabolic responses of heterogeneous cells.

In the vascular wall, gap junctions show a distribution in structures that link all of the different cell types.[23] Gap-junction proteins may also coordinate the mechanical contractions of smooth muscle cells to modulate the vasomotor tone of the vessel wall. Vasoconstriction and vasodilation waves travel rapidly along the vessel network because of the conduction through gap junctions of signals between endothelial or smooth muscle cells (or both).[24]

Vascular cell gap junctions are essentially composed of four connexins (Cxs 37, 40, 43, and 45) in various relative amounts, depending on species and vascular beds.[25-27] This mechanism of intercellular communication has a key role in a variety of vascular functions[22,23] and pathologies where proliferation and migration are important, such as atherosclerosis,[28] endothelial wound repair,[29] and regulation of angiogenesis.[30]

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) are a class of cholesterol-lowering drugs that are used for the prevention and treatment of atherosclerosis.[31] In vitro studies have demonstrated that statins inhibit the proliferation and migration of rat smooth muscle cells[32] and decreased the neointima formation in organ-cultured human saphenous vein in association with reduced matrix metalloproteinase-9 levels.[18,31] Recently, statins have been shown to reduce Cx43 expression in human vascular cells.[28]

No study to date has evaluated whether gap-junctional communication is involved in the process of intimal hyperplasia in human. This assertion was investigated by using the aforementioned organ culture model of intimal hyperplasia in human saphenous veins. Here, we show that the development of intimal hyperplasia was associated with changes in the expression of Cx43.

MATERIAL AND METHODS

Harvesting of human saphenous vein and vein culture. Our institutional review committee approved the study. Surplus 5-cm-long segments of nonvaricose saphenous vein were obtained from 20 patients (mean patient age, 60 ± 3 years; 20 men) undergoing a lower limb bypass surgery. The surplus saphenous veins were dissected by using a minimal touch technique and those used for the experiment were immediately stored in Krebs solution without calcium (in mmol/L: NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; $KHPO_4$, 1.2; $NaHCO_3$, 25; ethylenediaminetetraacetic acid [EDTA], 0.026; glucose, 11.1). Two-centimeter segments of the harvested veins were cut open longitudinally with luminal surface facing upwards. The segments were then pinned out onto a Mersilene mesh (Ethicon) in a Pyrex dish, containing a layer of Sylgard 184 resin (Dow Corning, Seneffe, Belgium) and were cultured at 37°C in a humidified atmosphere of 95% air, 5% carbon dioxide. The segments were immersed in about 5 mL of the culture medium (Medium 199 Earle; Biochrom KG) supplemented with 10% newborn calf serum (Gibco BRL) and 1% antibiotic-antimycotic solution (10,000 U/mL penicillin G, 10,000 U/mL streptomycin sulphate, 25 μg/mL amphotericin B, and 5 μg/mL gentamicin) (Gibco BRL). The medium was changed every 2 days for 14 days.[13,14] The segments were then rinsed with phosphate-buffered saline (PBS) and snap frozen. Fluvastatin (Novartis) was used at a target molarity of 1 μM.

Histologic and histomorphometric analysis. Three 5-mm segments of vein were harvested before and after culture. After fixation in 4% formalin, the vein segments were paraffin embedded and 5-μm-thick sections were analyzed. Hematoxylin and eosin staining was used for histologic analysis, and van Gieson-elastin staining was used for histomorphometric assessment. For histomorphometry, the images were first digitalized and histomorphometric measurements were performed with specially designed software (KS 400, Zeiss, Germany) using a standardized protocol: 24 measurements of the intimal and medial thickness, evenly distributed across the whole section, were processed on each of two consecutive sections of each segment at a magnification of ×25.

RNA isolation and polymerase chain reaction. Fragments of the human saphenous vein were homogenized in the Tripure Isolation Reagent (Roche Diagnostics, Switzerland) using a Kinametic Polytron blender (Kinametica, Switzerland), and total RNA was extracted using the kit procedure. For real-time polymerase chain reaction (RT-PCR), total RNA was treated for 30 minutes in the presence of DNase I (DNA-free kit, Ambion, Cambridge, UK) and reverse-transcribed using ImProm-II Reverse Transcription System (Promega, Switzerland). The PCR reaction was performed in the presence of 20 ng sense- and antisense-specific primers using recombinant *Taq* DNA polymerase (Gibco-BRL).

The products obtained after 30 PCR cycles performed in a PCR machine (Biometra, Switzerland) with the different primers were visualized after ethidium bromide staining on agarose gel. Negative controls included amplification of distilled water and human vein RNA samples that had not been reverse-transcribed. The following primers were used:

- human Cx43 (649 bp fragment): 5′-GAACTCAAG-GTTGCCCAAAC-3′ (sense primer) and 5′-TTA-GAGATGGTGCTTCCCG-3′ (antisense primer);
- human Cx40 (416 bp fragment): 5′-TGGAGGT-GGGCTTCATTGTG-3′ (sense primer) and 5′-TACTTGCTCGGTGACCAGGTTG-3′ (antisense primer);
- human Cx37 (718 bp fragment): 5′-ACGAGCAGT-CAGATTTCG-3′ (sense primer) and 5′-GGATGA-GAGCCCATTGTAG-3′ (antisense primer);
- human Cx45 (570 bp fragment): 5′-GATTGC-CAAAATGGAGCACG-3′ (sense primer) and 5′-AGGGGGAGCAGATGGTGTATTC-3′ (antisense primer); and
- human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (114bp): 5′-AACTTTGGTATCGTG-GAAGG-3′ (sense primer), and 5′-CAGTAGAGG-CAGGGATGATGT-3′ (antisense primer).

Quantitative RT-PCR was performed on total RNA treated 30 minutes in the presence of DNase I (DNA-free kit, Ambion, Cambridge, UK) by using a LightCycler instrument (Roche Diagnostics GmBbH, Mannheim, Germany) and the QuantiTect SYBR Green PCR Kit (Qiagen, Basel, Switzerland). One-microgram aliquots of DNase-treated RNA were reverse-transcribed using ImProm-II Reverse Transcription System (Promega), as described in the kit protocol. The cDNA samples were subjected to serial dilutions in water. Each reaction mixture (20 μL) contained 4 μL cDNA, 10μL 2× PCR Master Mix (containing HotStarTaq DNA Polymerase, buffer, and dNTPs) and 3 pmol of each primer.

The amplification program consisted of one cycle of 15 minutes at 95°C, followed by 45 cycles of 15 seconds at 95°C, 20 seconds at 50° to 52°C, and 20 seconds at 72°C. In each run, contamination and specificity of the PCR was checked by including both a nonreverse-transcribed RNA sample and a sample of 2× PCR master mix containing 3 pmol of each primer, respectively. Amplification was followed by melting curve analysis to verify the identity of the amplicon.

PCR efficiency was determined by analyzing a dilution series of the RT-PCR reaction that contained the target gene and that served as a reference standard. Analysis of data was performed using the 3.5 version of the Light Cycler software (Roche Diagnostics GmbH, Germany), which generated a best-fit line from the log-linear region of each curve defining the crossing line. The intersection between the emitted fluorescence and the crossing line defined the crossing point. The concentration of target DNA was calculated by plotting the crossing point of each sample on the standard curves. The cDNA was amplified using the following primers:

- human Cx43 (123 bp): 5′-TTTCAATGGCTGCTC-CTC-3′ (sense primer) and 5′-TGCTCACTTGCTT-GCTTGTT-3′ (antisense primer);
- human β_2 microglobulin (100bp): 5′-TGAGTATGC-CTGCCGTGTGA-3′ (sense primer) and 5′-GGCATCTTCAAACCTCCATG-3′ (antisense primer); and
- human GAPDH (114bp): 5′-AACTTTGGTATCGTG-GAAGG-3′ (sense primer) and 5'-CAGTAGAGG-CAGGGATGATGT-3' (antisense primer).

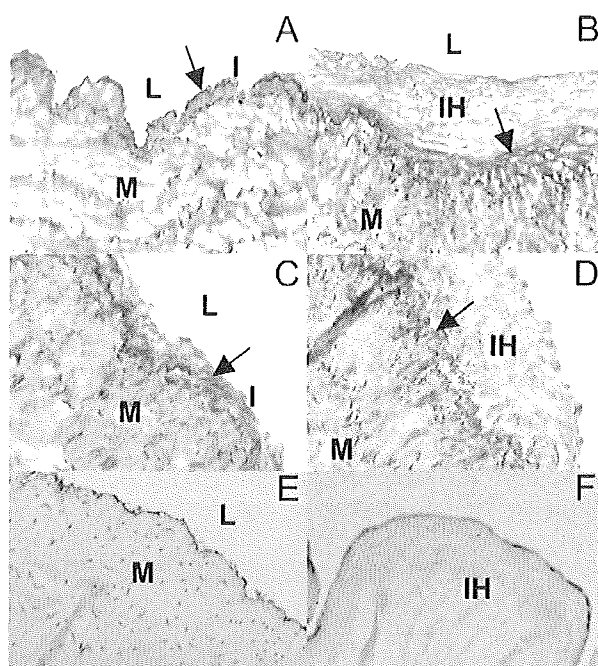
Data were analyzed with the LightCycler Software (Roche, Roche Diagnostics).

Western blotting. Fragments of human saphenous vein were rapidly frozen in liquid nitrogen. Each vein was pulverized with a pestle and mortar precooled in liquid nitrogen, and the powder was homogenized and sonicated in 62.5 mM Tris(hydroxymethyl)aminomethane (pH 6.8), 5% sodium dodecylsulfate, and 10 mM EDTA. Protein content was determined by Bio-Rad DC Protein Assay Reagent Kit (Bio-Rad Laboratories).

Aliquots of veins were heated at 50°C in loading buffer, fractionated by electrophoresis in a 12.5% polyacrylamide gel, and immunoblotted overnight onto Immobilon polyvinylidene fluoride membranes (Millipore Co) at a constant 20V. Membranes were incubated for 1 hour in PBS containing 5% milk and 0.1% Tween 20 and then incubated overnight at 4°C with an antiserum against Cx43 (antirabbit polyclonal antibodies, AB1728, Chemicon) or Cx40 (antirabbit poyclonal antibodies, AB1726, Chemicon) diluted 1:200, tubulin, or β-actin antihuman smooth muscle cells (Sigma, St Louis, Mo) diluted 1:2000 in blocking buffer.

After the immunoblots were rinsed in PBS containing 0.1% Tween 20, they were incubated for 1 hour with anti-rabbit immunoglobulin (Ig) antibody coupled to alkaline phosphatase (Dako Diagnostic AG) that was diluted 1:5000. Antigen-antibody complexes were detected with the HRP Western blot detection system (Amersham). In these experiments, controls included use of rat lungs and bovine aorta endothelium.

Immunofluorescence and histochemistry. The human saphenous veins were cut in fragments that were quickly frozen in 2-methylbutane precooled in liquid nitrogen. Vein fragments were frozen in optimal cutting temperature medium (Miles Inc) and cryo-sectioned at about 5 μm thickness. Sections were rinsed in PBS, incubated 30 minutes in a buffer containing 0.5% bovine serum albumin (BSA) and 0.2% Triton X-100, and then exposed for 20 hours to antibodies against either Cx43 (AB1728, Chemicon), or Cx40 (AB1726, Chemicon) and α-smooth muscle cell actin (A2547, Sigma) diluted 1:500 in PBS. Primary antibodies were detected using secondary antibodies labelled with fluorescein isothiocyanate (Molecular Probes Inc) and directed against rabbit IgG. Sections were then rinsed in PBS, counterstained with Evans Blue and cover-



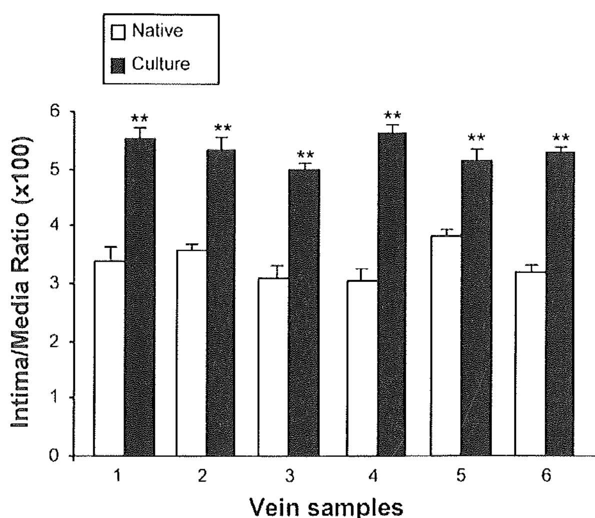


Fig 1. Upper panel, Development of intimal hyperplasia in human saphenous vein segments after 14 days in culture. **A,** Compared with control vein, **(B)** intimal and subintimal space thickness, corresponding to intimal hyperplasia (IH) is markedly increased (van Gieson-elastin staining) in human venous segment after 14 days in culture. **C** and **D,** Immunolabeling of the sections shown in **A** and **B,** with antibodies against α-actin (brown color) revealed the presence of smooth muscle cell proliferation in the neointima (*arrows* in A, B, C, and D, represent the lamina elastica interna). **E** and **F,** Immunolabeling with antibodies against the factor VIII demonstrated the presence of endothelial cells along the vessel. Original magnification, ×400 in **A, B, C, D** and ×200 for **E** and **F.** *L,* Lumen; *I,* intima; *IH,* intimal hyperplasia; *M,* media. **Lower panel,** Quantitative assessment of six measurements (one per human vein segment) of intimal and subintimal space thickness, corresponding to intimal hyperplasia, was significantly increased in vein segments incubated in culture for 14 days compared with controls *(Native);* $**P < .01$.

slipped. Paraffin embedded sections (5 μm) were also immunolabeled with antibodies recognizing von Willebrand factor (Dako diluted 1:1000) or the smooth muscle cells β-actin (diluted 1:1000) (A5441, Sigma). Sections were viewed on a microscope (Leica).

Statistical analysis. Data were expressed as mean ± SEM. Differences between means were assessed with the Student's t test, analysis of variance, or both. Statistical significance was defined at a value of $P < .05$, $P < .01$, and $P < .001$.

RESULTS

An in vitro model of human saphenous vein hyperplasia. Intimal hyperplasia was induced experimentally using an ex vivo organ culture model of human saphenous veins. Veins were incubated in culture for a period of 14 days. The integrity of the vessel wall was maintained during the culture period (Fig 1, *A* and *B*). The presence of smooth muscle cells and endothelial cells was demonstrated using immunoreactivity for α-actin (expressed in smooth muscle cells, Fig 1, *C* and *D*) and for von Willebrand factor (expressed by endothelial cells, Fig 1, *E* and *F*). Quantitative assessment of histomorphometric measurements from van Gieson-elastin stained sections of six different venous segments before and after vein culture during a period of 14 days demonstrated a significant increase of the ratio between intimal and medial thickness compared to the respective freshly isolated vein (Fig 1, lower panel).

Increased connexin43 in human saphenous vein in culture. RT-PCR analysis of freshly isolated human vein mRNA demonstrated the presence of a transcript for four connexins (Cx37, Cx40, Cx43, and Cx45) but not for Cx36 (data not shown). After 14 days in culture, the transcript for Cx43 was increased and a similar expression pattern was observed for the Cx37, Cx40 and Cx45 mRNAs compared with controls (Fig. 2, *A*). In view of previous reports on human[33] and rabbit[34] vascular smooth muscle cells, we focused our experiments on Cx43, thought to be the prominent connexin in smooth muscle cells.

Quantitative RT-PCR evaluation of Cx43 mRNA expression normalized with β_2 microglobulin or GAPDH (not shown) in human saphenous vein in culture demonstrated that hyperplasia was associated with a large increase in the levels of Cx43 mRNA after 14 days in culture (Fig 2, *B*).

Western blot analysis of total human vein extracts showed that Cx43 was expressed as two major immunoreactive forms, with apparent molecular weights of 43 and 44 kDa. Comparison of multiple independent samples showed that the levels of Cx43 were significantly increased in the vein after 14 days in culture. Quantitative evaluation of the two immunoreactive bands revealed that after 14 days in culture, the levels of Cx43 were increased ($P < .001$) about eightfold (Fig 2, *C* and *D*). The expression of Cx43 rose between 1 and 3 days in culture, and was greatest at 14 days (Fig 3). Throughout this period, there was no change in the levels of Cx40 (Fig 3).

Immunolabeling demonstrated that Cx43 was immunolocated on smooth muscle cells of freshly frozen human

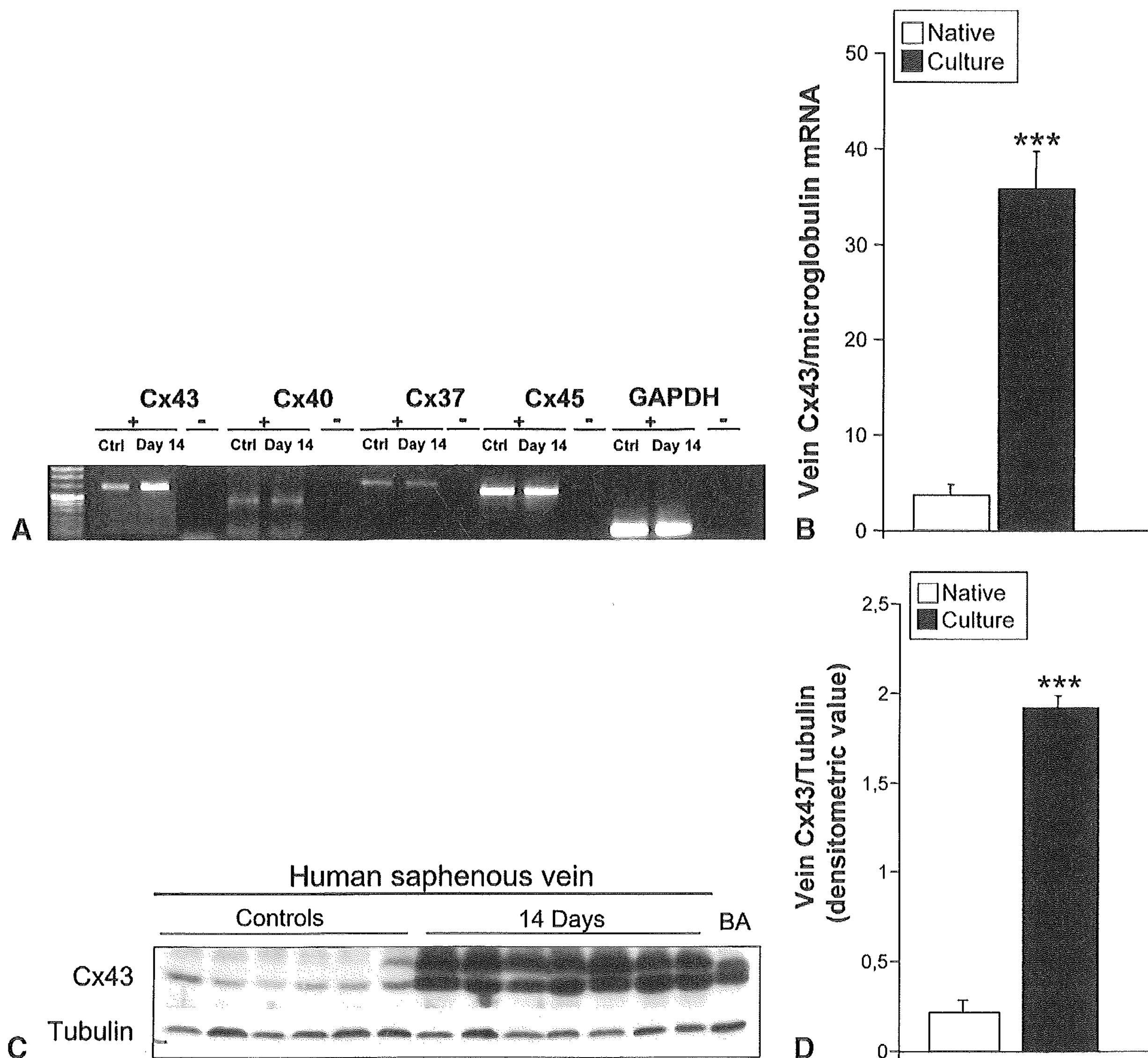


Fig 2. Connexin43 (Cx43) is increased at both mRNA and protein levels in veins after 14 days in culture. **A,** Multiple connexin transcripts are expressed in the human saphenous vein wall. Reverse-transcribed human saphenous vein RNA was amplified by polymerase chain reaction (PCR) using primers specific for human Cx37, Cx40, Cx43, and Cx45. The amplification of four amplicons corresponding to Cx37, Cx40, Cx43, and Cx45 are shown after ethidium bromide staining. These products were not amplified in samples that were not reverse-transcribed before PCR processing *(–)*. This experiment was performed in triplicate with different human vein segments. **B,** Expression of Cx43 mRNA analyzed by quantitative real-time PCR is increased in vein that is developing intimal hyperplasia. Quantitative assessment of six to seven measurements (one per human vein segment) showed that Cx43 mRNA was increased sixfold in vein segments cultured during a period of 14 days compared with control veins *(Native)*. Values represent ratios between Cx43 and β_2-microglobulin mRNAs expression. Data are expressed as mean ± SEM; $^{***}P < .001$. *GAPDH,* glyceraldehyde-3-phosphate dehydrogenase. **C** and **D,** Western blot analysis of total proteins showed that the levels of Cx43 were markedly increased (about 10-fold) in vein after 14 days in culture compared with control levels. Data are expressed as mean ± SEM; $P < .001$. *BA,* bovine aorta. Under both conditions, Cx43 was detected as two bands of 43-44 kDa. Each lane represents a sample from a different vein. All lanes were loaded with 75 μg total proteins. In the same samples, levels of tubulin were not modified. As positive control, 25 μg of freshly scraped bovine aortic endothelium was used.

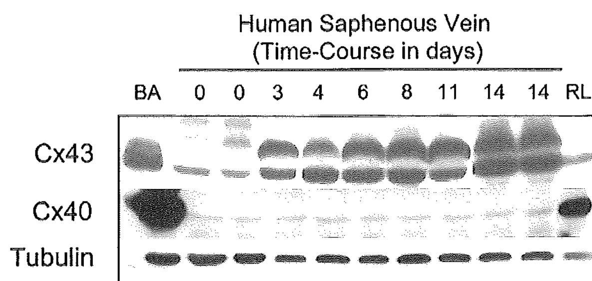


Fig 3. Cx43 protein levels change during the development of intimal hyperplasia in human saphenous vein. Western blot analysis of total proteins content revealed that the expression of connexin43 (Cx43) in venous samples progressively increased during the first 3 days of organ culture. In the same samples, no change in the expression of Cx40 was observed during culture period. All lanes were loaded with 75 μg of total proteins. Each lane represents a sample from a different vein. As positive controls, 25 μg of bovine aorta *(BA)* endothelial proteins and 40 μg of rat lung *(RL)* proteins were used. Levels of tubulin proteins were not modified.

saphenous vein (Fig 4 *A*, upper panel). After 14 days in culture, a marked increase in the number of immunoreactive dots was observed in the smooth muscle cells (Fig 4, *E* and *G* upper panel). Fig 4, *C* and *G* (upper panel) also showed Cx43 expression on both smooth muscle cells and endothelial cells. Cx40 staining was only observed in the endothelial layer (Fig 4, *H* upper panel).

Immunofluorescence labeling of serial sections of the human saphenous vein before (Fig 4, *A* and *B* lower panel) and after 14 days in culture (Fig 4, *C* and *D* lower panel) was performed using specific antibodies against Cx43 and α-smooth muscle cells. These data revealed that the increase in the number of Cx43 immunofluorescent spots observed after 14 days in culture occurs mainly in the smooth muscle cells.

Reduction of intimal hyperplasia and Cx43 expression in organ culture by fluvastatin. We examined whether the cholesterol-lowering drug fluvastatin inhibited the neointima formation in organ-cultured human saphenous vein. Fluvastatin reduced neointimal thickness of about 50% ($P < .01$) (Fig 5, *A*) at a concentration of 1 μM known to have no detrimental effects on cell viability in saphenous vein organ cultures.[18,35] Western blot analysis of veins segments cultured for 14 days in presence of fluvastatin demonstrated a marked decrease in the levels of the two immunoreactive bands corresponding to Cx43 (Fig 5, *B*). Cx43 was significantly reduced by 50% to 70% in the veins incubated in presence of fluvastatin compared with controls ($P < .01$) (Fig 5, *C*).

DISCUSSION

Stenosis due to intimal hyperplasia is the most common cause of failure of venous bypass grafts. To better understand the development of intimal hyperplasia, we used saphenous veins harvested from patients undergoing a lower limb bypass surgery in an ex vivo organ culture model. This model maintains the morphologic and functional integrity of the vessel wall and a significant development of intimal hyperplasia occurs after 14 days in culture.

We have postulated that gap junctions, which coordinate physiologic processes such as cell growth and differentiation, may participate in the development of intimal hyperplasia. Indeed, intimal hyperplasia consists of proliferation and migration of smooth muscle cells into the subendothelial space. This is the first study that evaluates the involvement of gap junctional communication in the process of intimal hyperplasia in humans. Our data demonstrate a critical role for Cx43-mediated gap junctional communication in the human vein during the development of intimal hyperplasia in the aforementioned organ culture model.

Gap junction channels provide an enclosed conduit for direct exchanges of signalling molecules, including ions and small metabolites, between cells. This system of communication allows cells to review the functional state of their neighbors. In vessels, endothelial cells may express three connexin isotypes, namely Cx37, Cx40, and Cx43, whereas the underlying smooth muscle cells may express Cx37, Cx40, Cx43, and Cx45. In most vascular smooth muscle cells, Cx43 expression and function seem dominant.[36-38]

Our study investigated the changes in Cx43 expression that might accompany the development of intimal hyperplasia in an in vitro culture model of human saphenous vein. We showed that Cx43, but not Cx40, is markedly overexpressed during the 14-day culture period in the smooth muscle cells and that treatment with fluvastatin caused a reduction of neointima formation in cultured human saphenous vein segments in association with a marked decrease in Cx43 expression. These results provide evidence that increased Cx43 is associated with the in vitro development of intimal hyperplasia in the human vein.

Statins and marimastat have been shown to decrease intimal hyperplasia by reducing matrix metalloproteinase-9 activity and smooth muscle cell proliferation.[17] Recently, it has been demonstrated that statins reduced Cx43 expression in primary human vascular cells in vitro,[28] and in vivo studies have further shown an association between reduced Cx43 expression levels in mice treated with statins and the presence of a lower number of inflammatory cells in atheroma.[28] Although we used an in vitro organ culture model, it uses the exact tissue (saphenous vein) that causes the clinical problem in vivo and our observations provide the first report that statins reduced Cx43 expression in human vascular tissue.

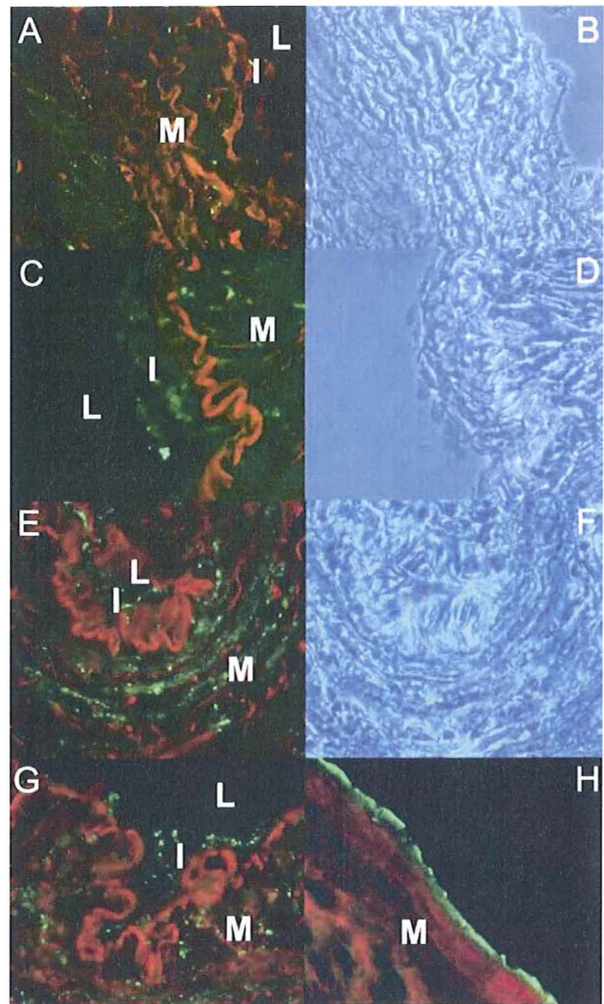
Communication via gap junctions has been implicated in the regulation of cell growth, differentiation[39] and migration.[29,30,40-42] These three processes have a central role in the development of intimal hyperplasia. Accordingly, we show that conditions that modify vein homeostasis due to changes in the proliferation and migration rate of smooth muscle cells are associated with a marked increase in Cx43 expression. During hyperplasia, the increase of Cx43 is associated with smooth muscle cell proliferation, and the

mechanism underlying the changes in Cx43 expression remains to be elucidated.

The finding that the changes in Cx43 expression took place within few days indicates that the increase in Cx43 expression in smooth muscle cells is an early event. Changes in Cx43 levels were also significantly faster than those required to observe structural wall modifications, such as smooth muscle cells hyperplasia and migration, suggesting that alterations of the normal connexin pattern may induce the subsequent structural and functional changes in the muscle layer.

The abundance of Cx43 suggests that gap junctional communication between smooth muscle cells may play a role in the initiation of the proliferative response and subsequently in maintaining it. The response of smooth muscle cells in the human vein initially involves replication in the media followed by migration to the neointima, and the question concerns the relationship between the upregulation of Cx43 and the development of intimal hyperplasia. Increasing evidence suggests that reduced gap junctional communication is involved in uncontrolled growth.[43-45] Our findings suggest, however, that upregulation of Cx43 in the human vein is linked to smooth muscle cell activation and may be a transient event in the cell cycle of the smooth muscle cell, as has already been observed during vascular healing in the rat carotid artery.[46]

Intimal hyperplasia is the process by which the cell population increases within the innermost layer of the arterial wall[47] such as occurs physiologically in homografts, transplanted organs,[48] and in veins used as arteriovenous fistulas or arterial bypass grafts.[49] It consists of proliferation of the vascular smooth muscle cells



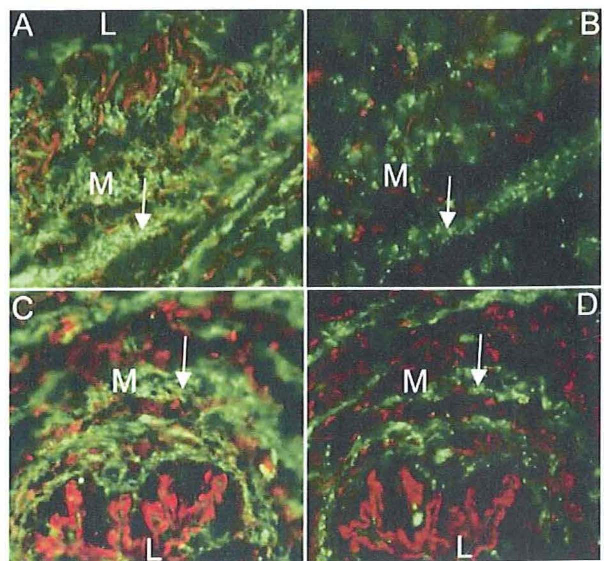


Fig 4. Connexin43 (Cx43) is increased in smooth muscle cells of human saphenous vein in culture. **Upper panel,** Phase-contrast views of sections of human saphenous vein before **(B)** and after14 days in culture **(D, F).** Immunofluorescence labeling with specific antibodies located Cx43 at discrete spots dispersed throughout the media of the vein wall **(A)**. A sizable increase in the number and pattern of these spots was noticed in human vein that was developing hyperplasia after 14 days in culture **(C, E)**. The limited amount of Cx43 expressed by the endothelial cells is visible in panel E. Cx43 expression in smooth muscle located in the intimal hyperplasia cells is visible on panel C and G. Immunofluorescence labeling with Cx40 antibodies reflected the presence of Cx40 only in the endothelial cells **(H)**. *L,* Lumen; *I,* intima; *IH,* intimal hyperplasia; *M,* media. Original magnification: ×400. Lower panel, Immunofluorescence labeling of serial sections of the human saphenous vein before **(A, B)** and after 14 days in culture **(C, D)**. Alpha-smooth muscle actin was localized in the smooth muscle cells of the human vein **(A, C)** and demonstrated that Cx43 is located in the human vein smooth muscle cells **(B and D)**. Cx43 and α-smooth muscle staining revealed that the increase in the number of Cx43 immunofluorescent spots observed after 14 days in culture occurred in the smooth muscle cells **(B, D)**. *Arrows* indicate the presence of α-smooth muscle actin in A and C and Cx43 in B and D. *L,* Lumen; *M,* media. Original magnification, ×400.

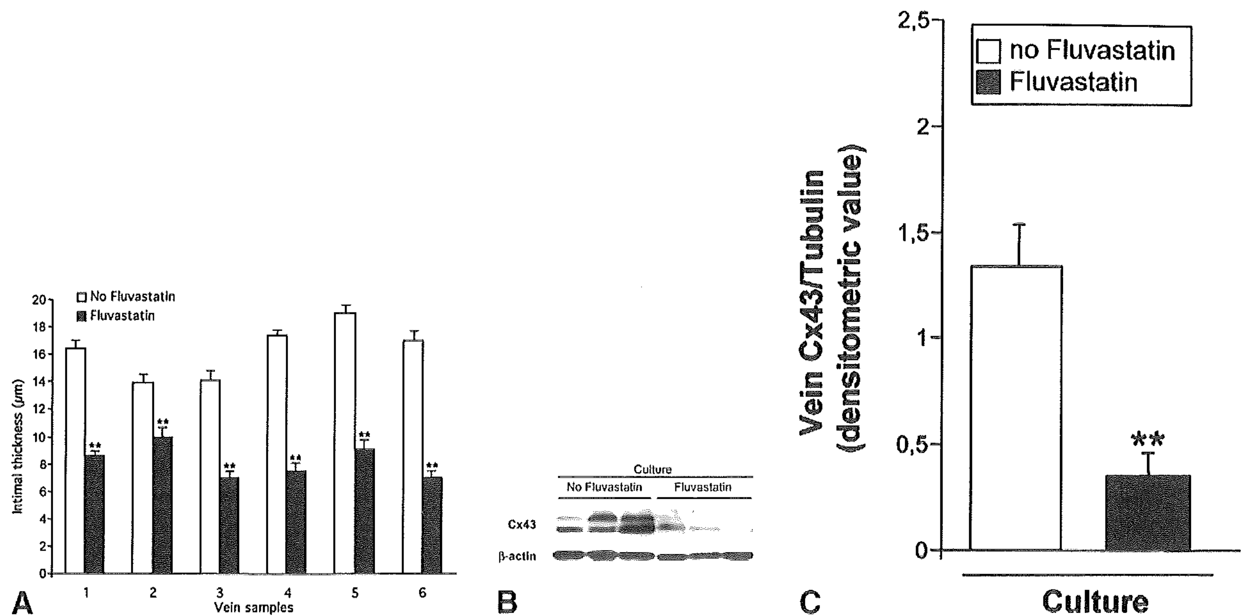


Fig 5. Fluvastatin decreased intimal hyperplasia and connexin43 (Cx43) levels in human saphenous veins in culture. **A,** Quantitative assessment of six measurements (one per human vein segment) of intimal thickness, corresponding to intimal hyperplasia, was significantly decreased in vein segments after 14 days in culture in the presence of fluvastatin compared with control veins cultured without fluvastatin. $^{**}P < .01$. **B,** Western blot revealed that the levels of Cx43 were decreased in vein segments incubated in the presence of fluvastatin compared with controls. **C,** Quantitative evaluation of six immunoblots (one per vein) revealed that the levels of Cx43 were decreased threefold to fourfold in veins incubated with fluvastatin. $^{**}P < .01$.

followed by their migration across the lamina elastica interna and their secretion of extracellular matrix in the subendothelial space.

The contractile smooth muscle cells of adult veins exhibit low rates of proliferation[50]; they are rarely called upon migrate to re-establish vascular integrity, but smooth muscle cells retain the ability to migrate and divide rapidly in response to injury. Such a change in behavior requires a switch in the spectrum of active genes involved for migration and proliferation[51] such as the metalloproteinase[31] and the nitric oxide synthase genes.[52] The marked increase of Cx43, already observed after a few days in cultured veins, suggests that Cx43 might play a role in the initiation step of proliferation and also during the formation of intimal hyperplasia.

Atherosclerosis, the most common pathophysiologic process, involves expansion of the intimal vascular smooth muscle cell population[53] together with the accumulation of lipids and leucocytes.[54] Evidence is growing that dysfunctional gap junctional intercellular communication is involved in the development of atherosclerosis.[55] Blackburn et al[56] reported that Cx43 expression in intimal smooth muscle cells was shown to increase at the early stages of human coronary atherosclerosis and to decrease at later stages of the disease.[56] Recently, it was demonstrated that the expression of Cx43, Cx40, and Cx37 is altered in atherosclerotic plaques[57] and that reduced Cx43 levels inhibit the formation of atherosclerotic lesions in low-density-lipoprotein receptor-deficient mice.[28] These data indicate that Cx43 has a role in atherosclerotic plaque formation, and a possible mechanism may involve Cx43-mediated effects on smooth muscle cell proliferation or function.[55]

To proliferate and migrate, vascular smooth muscle cells should undergo a phenotypic transformation, which is a key step in neointimal formation. From their resting, contractile state, they revert to a more embryonic phenotype that allows the synthesis of extracellular matrix. Inhibition of this change by transforming growth factor-β (TGF-β)[58] can prevent the development of intimal hyperplasia. It was recently demonstrated that the Cx43-mediated heterocellular gap junction between endothelial and mesenchymal cells is necessary for contact-dependent activation of TGF-β, which mediates endothelial-induced mural cell differentiation.[59] These data reinforce the hypothesis that increased Cx43 expression plays a role in the differentiation of smooth muscle cells.[38,60,61]

In conclusion, our findings represent an important contribution to the understanding of the mechanisms involved in the development of intimal hyperplasia by identifying Cx43 as a target protein that is markedly increased in intimal hyperplasia. These data also show that fluvastatin could prevent this induction, supporting a crucial role for Cx43-mediated gap junctional communication in the human vein during the development of intimal hyperplasia.

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