Impaired Notch4 Activity Elicits Endothelial Cell Activation and Apoptosis

Implication for Transplant Arteriosclerosis

T. Quillard, S. Coupel, F. Coulon, J. Fitau, M. Chatelais, M.C. Cuturi, E. Chiffoleau, B. Charreau

Objective—Notch signaling pathway controls key functions in vascular and endothelial cells (EC). However, little is known about the role of Notch in allografted vessels during the development of transplant arteriosclerosis (TA). This study investigated regulation of the Notch pathway on cardiac allograft arteriosclerosis and further examined its implication in EC dysfunction.

Methods and Results—Here we show that, among Notch receptors, Notch2, -3, and -4 transcript levels were markedly downregulated in TA compared to tolerant and syngeneic allografts. TA correlates with high levels of tumor necrosis factor (TNF), transforming growth factor (TGF)β, and IL10, which consistently decrease Notch4 expression in transplanted and cultured ECs. We found that inhibition of Notch activity, reflected by both a reduced CBF1 activity and Hes1 expression, parallels the downregulation of Notch4 expression mediated by TNF in ECs. Notch4 and Hes1 knockdown enhances vascular cell adhesion molecule-1 expression and promotes EC apoptosis. Silencing Notch4 or Hes1 also drastically inhibits repair of endothelial injury. Overall, our results suggest that Notch4 and basal Notch activity are required to maintain EC quiescence and for optimal survival and repair in response to injury.

Conclusion—Together, our findings indicate that impaired Notch4 activity in graft ECs is a key event associated with TA by triggering EC activation and apoptosis. (Arterioscler Thromb Vasc Biol. 2008;28:2258-2265.)

Key Words: transplant arteriosclerosis • notch • endothelial cells • activation • apoptosis

Transplant arteriosclerosis (TA) is the main limitation for long-term functioning of solid organ allografts. In TA, allograft arteries characteristically develop severe diffuse intimal hyperplasia that ultimately allows luminal stenosis and cause ischemic graft failure. Characteristics of the lesions include endothelial cell (EC) damage, mononuclear cell infiltration, smooth muscle cell (SMC) proliferation, and matrix protein deposition in the intima of the vessel wall. Neointimal formation begins only after EC loss followed by SMC recruitment at the sites of injury suggesting that endothelial injury or denudation is an important proximal step that initiate vasculopathy. Recipient alloreactive T cells and antibodies, as well as infiltrating macrophages and NK cells, can all contribute to EC apoptosis and vascular injury. Development of TA requires interferon (INF)-γ and other proinflammatory cytokines such as tumor necrosis factor (TNF). However, the precise pathogenesis of transplant arteriosclerosis is not fully understood.

Notch signaling pathway regulates a broad array of cell fates and cellular processes during embryonic development and contributes to adult homeostasis. Notch proteins comprise a family of transmembrane receptors and ligands highly conserved through Evolution. In mammals, 4 Notch receptors (Notch1–4) and 5 Notch ligands (Dll1,-3,-4, Jag1–2) have been identified. The binding of ligands to Notch receptors results in a series of proteolytic cleavages of the receptor managed sequentially by ADAM proteins (a desintegrin and metalloproteinase) and the γ-secretase complex. Generation of the cytoplasmic Notch C-terminal intracellular fragment (NICD) is followed by its translocation into the nucleus and forms a complex with the transcriptional repressor CBF1/RBP-Jk. NICD binding to CBF1/RBP-Jk induces coactivators recruitment and allows the expression of various lineage-specific genes.

Evidence that the Notch pathway plays a critical role in vascular development and homeostasis includes the specific expression of Notch pathway ligands and receptors in vascular ECs and SMCs, as well as the phenotypes of knock-out mice for several Notch components. Expression analyses revealed that the major components of the Notch pathway in the vascular system consist of 4 ligands (Dll1, Dll4, Jag1, and Jag2), 3 receptors (Notch-1, -3, and -4), and target family genes Hes and Hey. Among Notch receptors, only Notch4 displays an expression pattern restricted to endothelium.

Original received April 15, 2008; final version accepted September 3, 2008.

From INSERM, U643, Nantes, F44000 France; CHU Nantes, Institut de Transplantation et de Recherche en Transplantation, ITERT, Nantes, F44000 France; Université de Nantes, Faculté de Médecine, Nantes, F44000 France.

Correspondence to B. Charreau, INSERM U643, CHU Hôpital Dieu, 30, bd Jean Monnet, 440093 Nantes cedex 01, France. E-mail Beatrice.Charreau@univ-nantes.fr

© 2008 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.108.174995

2258
Combined deletion of Notch1 and Notch4 genes enhances the defects in vasculature remodeling observed in Notch1 single knock-out mice.\textsuperscript{11} EC-specific expression of an activated form of Notch4 led to embryonic lethality with abnormal vessel structure and patterning.\textsuperscript{12} Constitutive activation of Notch4 in ECs also causes defects in vascular remodeling.\textsuperscript{13} In vitro experiments also demonstrate that Notch4 activation protects ECs from apoptosis, promotes endothelial-to-mesenchymal transdifferentiation, and blocks both proliferation and angiogenesis.\textsuperscript{14–17}

Although the importance of Notch in controlling EC proliferation, differentiation, and survival has been established, little is known about the role of Notch expressed in allografted vessels during the development of transplant arteriosclerosis. This study investigated expression of Notch receptors in a fully MHC mismatched combination of rat cardiac allografts that elicits TA and further examined regulation of Notch signaling by silencing Notch4 and Hes1 in cultured vascular ECs. Taken together, our findings indicate that impaired Notch4 activity elicits TA by triggering EC activation and apoptosis.

**Methods**

**Cardiac Allografts**

Rats heterotopic cardiac allografts were performed with fully mismatched LEW.1W (RT1u) or LEW.1a (RT1a) as donors and LEW.1a as recipients (Centre d’Elevage Janvier). Recipients underwent either a donor-specific blood transfusion (DST) 7 and 14 days before transplantation or a 20-day course of the deoxyspergualine analogue LF015–095. At day 100 posttransplantation, clear-cut histological signs of TA were found in DST-treated animals whereas LF015–095–treated animals tolerated the graft without any signs of TA. Rat cellular subsets were isolated from Lewis Rats.

**Cell Culture**

Primary cultures of human arterial ECs (HAECs) and human umbilical vein ECs (HUVECs) were isolated and grown in ECBM with 10% fetal calf serum and supplement kit (Promocell).

**Transfections, CBF1-Luciferase Reporter Assay, and siRNAs**

The luciferase reporter plasmid that contains 4 copies of a binding site for CBF1 (CBF1-Luc) was transfected in HUVECs with lipo-2000 reagent (Invitrogen). For the positive control, cells were also transfected with a plasmid encoding Notch2 NICD. SiRNA duplexes purchased from Ambion were transfected with RNAiMAX lipofectamine reagent (Invitrogen).

**qRT-PCR, Western Blot, Immunohistochemistry Analysis, and Flow Cytometry**

Please see the supplemental data section, available online at http://atvb.ahajournals.org, for additional details.

**Scratch Assay & Time Lapse**

Confluent cell layers were scratched with a 20 μL micropipette tip and subjected for time-lapse video microscopy for 24 hours in complete culture medium.

**Cell Viability and Apoptosis Assays**

**MTT Assay**

HAECs were incubated with MTT for 4 hours. After DMSO addition, OD for each condition was measured at 570 nm.

**AnnexinV Staining**

ECs were stained with activated protein APC-conjugated-annexinV (BD Biosciences). Fluorescence was measured using a FACScalibur (BD Biosciences) and analyzed using FlowJo® software (Tree Star Inc).

**Statistics**

Presented data are representative for at least 3 independent experiments. Results are means±SE. Statistical analysis was performed by the parametric analysis of variance test. \( P<0.05 \) was considered statistically significant.

**Results**

**Downregulation of the Notch Pathway on Transplant Arteriosclerosis**

Firstly, expression of Notch receptors was examined in cardiac allografts at D100 posttransplantation. In this experimental model, donor-specific blood transfusions (DST) before transplantation allows long-term survival (>100 days) associated with TA whereas graft tolerance (TOL) is induced by a short-term treatment with a deoxyspergualine analogue (LF15–0195).\textsuperscript{18,19} Among Notch receptors, Notch2, Notch3, and Notch4 transcripts levels were markedly downregulated in TA allografts compared to TOL (13.3-, 53.8-, and 2.9-fold decrease, respectively) and to syngeneic controls (A/A; Figure 1). No significant change in Notch1 was observed. TA also correlates with a significant increase in adhesion molecule VCAM-1 expression suggesting that TA is associated with both an altered Notch signaling and EC dysfunction. Importantly, Notch4 expression assessed by Western blotting was reduced in the TA compared to TOL and syngeneic controls (Figure 1B). Quantification revealed that Notch4 expression was reduced by 55% in TA compared with syngeneic transplants (Figure 1C). Among Notch receptors, only Notch4 was reported as an endothelial-restricted molecule.\textsuperscript{10} We observed that Notch4 immunoreactivity was confined to the endothelial cell layer (mostly in arteries) in cardiac sections and overlap with PECAM-1 immunoreactivity. Immunohistological staining of Notch4 in allografts attested endothelial Notch4 decrease in arteries for both early and late arteriosclerotic lesions (Figure 1D). Moreover, analysis in rat cell subsets (EC, B-cells, T-cells, NK cells, macrophages, DCs: both resting and activated) confirmed that Notch4 was mostly expressed by EC (see supplemental Figure 1).

**Notch4 Expression Is Decreased in Vascular ECs by Inflammatory Cytokines Implicated in the Pathogenesis of TA**

Consequently, the role of the endothelial Notch4 signaling in arterial injury and remodeling was further investigated in cultured human vascular ECs. To develop a relevant in vitro model, we sought to characterize the pattern of inflammatory soluble mediators potentially implicated in the regulation of Notch pathway associated with TA.\textsuperscript{20,21} We showed that TNF, TGF–β, and IL10 expression was markedly increased in allografts with TA at D100 posttransplantation (2.5-, 3.6-, and 34.0-fold as compared to syngeneic transplants, respectively) (Figure 2). No significant change was observed at D100 in tolerant allografts (data not shown). Next, cultures of arterial ECs were treated with recombinant human TNF,
TGFβ, IL10 for various period of time. Notch4 expression was then analyzed by Western blotting. As shown in Figure 2B, TNF, TGFβ, and IL10 induced a strong time-dependant decrease in Notch4 expression. Moreover, TGFβ and IL10 displayed a major effect at 24 hours, whereas TNF has a maximal effect on Notch4 at 12 hours that is persistent up to 72 hours (Figure 2C). Western blotting analysis showed no significant change in Notch1 in endothelial cells stimulated with TNF for 2 to 24 hours (Figure 2D), suggesting that Notch1 expression does not compensate for the loss of Notch4 in response to TNF. Thus soluble mediators of inflammation implicated in TA directly affect Notch signaling pathway by decreasing endothelial Notch4 expression.

**Endothelial Decrease in Notch4 Is Associated With Canonical Notch Activity Reduction**

Canonical Notch activity allowing expression of Hes/Hey effector genes in a CBF1-dependent manner may be the
resultant of the combined Notch activation pattern. To test whether decrease in Notch4 expression by proinflammatory mediators of TA in ECs reflects a global decrease in Notch activity, ECs have been transfected with a luciferase reporter plasmid that contains 4 copies of a binding site for CBF1 (CBF1-Luc) and then treated with TNF. Figure 3A shows that TNF significantly decreases CBF1 promoter activity in ECs at 12 hours and provides a maximal inhibition at 24 hours (66 ± 18% and 94 ± 6% of decrease, respectively). ECs cotransfected with both CBF1/luciferase construct and a Notch2 NICD plasmid (N2IC/MSCV) were used as a positive control. Our findings also indicates that resting ECs display a constitutive Notch activity. To validate this observation, ECs were treated with DAPT, a specific γ-secretase inhibitor. We found that DAPT display a dose-dependent decrease in CBF1/luciferase activity with a maximal inhibition achieved at 10 μmol/L (80 ± 11% of inhibition versus untreated ECs) (Figure 3B) confirming that inhibition of γ-secretase efficiently abrogates Notch basal activity in resting ECs. Notch pathway activity can also be assessed by measuring Hes/Hey gene transcription. To confirm our results obtained with the CBF1 reporter assay, we further analyzed transcript level for Hes1 gene in ECs treated with TNF. In Figure 3C, decrease in basal Notch4 expression in response to TNF was also associated with a progressive and strong decrease of Hes1 mRNA level. Our results also show that regulation of Notch4 expression and overall Notch activity in ECs by TNF were both dependent on NFκB (see supplemental Figure II).

Silencing of Notch4 Is Sufficient to Impair Notch Activity in ECs

Parallel regulations for Notch4 expression and Notch activity by TNF strongly suggest that Notch4 could be responsible for the global Notch activity in ECs. To address this point, ECs were transiently transfected with 2 sets of siRNA specifically targeting Notch4. Knock-down was achieved and attested by qRT-PCR (95.3 ± 1.7% and 74.4 ± 6.9% decrease in mRNA level for Notch4 siRNA#1 and #2, respectively, as compared to scramble siRNA) without any affecting other Notch receptors (Figure 4A and supplemental Figure III). To test whether Notch4 inhibition impacts the overall Notch activity, silenced ECs were either collected to measure Hes1 expression or CBF1 activity after transfection of the CBF1 reporter construct. As shown in Figure 4B, Notch4 silencing by both siRNA duplexes efficiently downregulates Hes1 expression (73.0 ± 0.8% and 58.3 ± 3.2% for si#1 and si#2, respectively, as compared to scramble siRNA). CBF1-reporter assay indicated that Notch activity was also severely impaired after Notch4 siRNA transfection (97.9 ± 1.0% and 75.6 ± 1.0% for si#1 and si#2, respectively, as compared to scramble siRNA), suggesting that in ECs Notch activity directly relies on Notch4 expression (Figure 4C).

Silencing of Notch4 and Hes1 Promotes EC Activation and Apoptosis

Silencing targeting Notch4 or Hes1 was performed to determine how decrease in Notch4 and Hes1 expression by inflammatory cytokines could elicit EC dysfunction. Firstly, the impact of siRNA-mediated knockdown of Notch4 and Hes1 on EC phenotype was examined by flow cytometry (Figure 5A). Our data show that in the absence of TNF, Notch4 and Hes1 knockdown significantly induce VCAM-1 expression in ECs (2.0- and 1.6-fold increase versus scramble, for Notch4 and Hes1, respectively). De novo expression...
of E-selectin was also observed (data not shown). In addition, while TNF efficiently induces VCAM-1 expression, concomitant silencing of either Notch4 or Hes1 further enhances VCAM-1 expression (3.5- and 2.8-fold increase, as compared to scramble siRNA). In addition a metabolic viability assay indicates that Notch4 and Hes1 silencing dramatically decreases cell viability (31.8 ± 1.2% and 34.2 ± 3.5% for Notch4 and Hes1 knockdown as compared to controls, respectively; Figure 5B). Results were confirmed by DNA content analyses (data not shown). As shown in Figure 5C, inhibition of Notch4 and Hes1 expression strongly increases the frequency of AnnexinV-positive cells (6.8- and 6.0-fold increase, respectively) indicating that EC death results from apoptosis.

Silencing of Notch4 and Hes1 Inhibits Repair of Endothelial Injury

EC apoptosis triggers vascular remodeling that ultimately could leads to TA.1-4 Cell migration and proliferation are key processes controlling vascular remodeling. Thus we used a more integrative model to test whether Notch4 could play a role in endothelial monolayer repair after injury. To this aim, EC monolayers were wounded 24 hours after siRNA transfection, and wound healing was monitored by time-lapse imaging for 24 hours. Representative phase contrast micrographs illustrating repair of endothelial injuries are shown in Figure 5D. Wound area was calculated to allow wound closure quantification. Equal wound closure motility was observed for ECs transfected without siRNA (NT) or with scramble siRNA. In contrast, wound healing capacity of cells transfected by siRNA duplexes targeting either Notch4 or Hes1 was impaired by 22.5 ± 2.1% and 27.0 ± 1.3% compared to scramble control, respectively.

Discussion

The precise pathogenesis of graft arteriosclerosis is unknown.1,2,4 Given the role of Notch signaling in vascular morphogenesis and remodeling,8 we speculate that a better understanding of Notch functions in transplant vascular injury might lead to the development of new therapeutic or diagnostic strategies.

In the present study, we demonstrated that allograft accelerated-transplant arteriosclerosis associates with a specific pattern of expression for Notch receptors. Decrease in Notch2, -3, and -4 features TA at D100 after cardiac
transplantation and correlates with an increased level of VCAM-1 expression, an adhesion molecule mainly expressed on activated vascular ECs. Moreover, TA also correlates with high levels of proinflammatory cytokines including TNF, TGFβ, and IL10 as previously reported. In contrast, no significant changes in Notch receptors were found in long-term surviving (ie, tolerant) allografts without TA as compared to syngeneic controls.

The vascular endothelium expresses all 4 Notch receptors, but only Notch4 displays an almost exclusively endothelial expression pattern whereas Notch1–3 are expressed more broadly. Therefore, we speculate that regulation of Notch4 in ECs may play a role in vascular lesions and remodeling that trigger TA. Decrease in Notch4 was consistently obtained in cultured ECs in response to TNF, TGFβ, and IL10, pointing out a functional relevance of Notch signaling in inflammatory processes. Notch activation also occurs in macrophages on inflammation and Notch components are present within macrophages in atherosclerotic plaques. Differential Notch4 regulation has been reported according to vascular sites and development stages that might reflect specific signaling mediated by cytokines and growth factors. Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2) modestly increase Notch1 and Notch4 mRNA in HUVECs. Cerivastatin, an antiangiogenic factor, downregulates Notch4 mRNA in FGF-2–treated ECs. TNF induces Notch4 mRNA in arthritic, but not normal, synovial fibroblasts; however, mechanisms underlying transcription of Notch4 remain largely unknown. Whether partial loss of ECs and their associated Notch4 molecules in arteriosclerosis lesions could also account for the observed decreased Notch4 levels observed in TA, in addition to the downregulation of

**Figure 5.** Effect of Notch4 and Hes1 silencing on EC activation, apoptosis, and wound repair. A, VCAM-1 induction analyzed by FACS after activation with or without TNF. B, EC viability was quantified using an MTT assay. C, Cell Apoptosis study was managed by FACS after staining with Annexin V. D, siRNA-transfected ECs were subjected to scratch assay. Wound closure was followed for 24 hours by time lapse imaging (*P<0.05 vs scramble control).
ECs Notch4 mRNA expression via exposure to inflammatory cytokines, could not be excluded. However, this hypothesis is not fully supported by immunohistochemistry showing sustained PECAM-1 immunoreactivity in TA (Figure 1D).

We have used 2 readouts to demonstrate a basal and significant activity of Notch pathway in quiescent ECs. The decreased activity of CBFI reporter assay in response to a γ-secretase inhibitor (DAPT) and the constitutive expression of effector target gene Hes1 both indicate that Notch pathway is basally activated in quiescent EC monolayers as also suggested by previous studies. Importantly, our findings further demonstrate that TNF provides a time-dependent decrease in Notch activity reflected by both a reduced CBFI reporter assay in response to a TNF/HD-H11022/B-dependent mechanism.

A similar decrease in Hes1 transcription in response to TNF has been reported previously in fibroblast and epithelial cell lines. Interestingly, NFκB activation was shown to enhance constitutive Hes1 expression in early time points but did not alter late (>1 hour) TNF-dependant repression of Hes1 transcription. Using chemical inhibitors PDTC and NAC, we established that concomitant suppression of Notch4 and Notch activity by TNF after 24 hours were both dependent on NFκB pathway, suggesting that decrease in global Notch activity rely on Notch4 expression rather than a direct NFκB-dependent mechanism.

While our data confirm a role for inflammatory cytokines in the regulation of Notch pathway, they also address the mechanism by which regulation of Notch signaling may affect EC functions. ECs are a common cellular target of all initiating events (allo-immune response, inflammation, and immunosuppressive drug cytotoxicity) implicated in TA development. Resultant chronic EC activation, apoptosis, and injury may play a pivotal role in vascular remodeling. The recruitment of inflammatory cells into allografts requires adhesion to activated vascular cells and endothelial expression of several adhesion molecules (eg, intercellular adhesion molecule intercellular adhesion molecule-1 (ICAM-1), VCAM-1, E/P-selectin) correlates with both acute rejection and TA. We demonstrate that Notch signaling also controls adhesion molecules, at least VCAM-1 and E-selectin, that are critical in mediating leukocyte adhesion and infiltration, but also in mediating SMC migration, both events that trigger arteriosclerotic lesions. By activating but also drastically enhancing EC activation on TNF treatment, decreased Notch signaling could greatly contribute to the maintenance of EC dysfunction within the graft that promote vascular remodeling and ultimately graft failure.

In ECs, constitutive expression of the active domain (NICD) of Notch4 blocks cell cycle and promotes from apoptosis. Consistent with these data, silencing Notch4 promotes cell death and impairs injury repair processes without any visible effect on proliferation (data not shown). Notch activity blockade through Hes1 knockdown showed similar effect, indicating that Notch4 functions were mediated by Hes1. Our data are consistent with approaches targeting Notch signaling by the use of γ-secretase inhibitors which showed impaired wound healing process in keratinocytes or fibroblasts and cell apoptosis induction in Kaposi sarcoma or in multiple myeloma. Surprisingly, as Notch signaling has been so far implicated in the regulation of sprouting angiogenesis and arteriovenous differentiation, no effects on endothelial viability have been observed in Notch4-deficient mice. Endothelial-specific Notch1−/− mice exhibit a similar phenotype to global Notch1−/− mice, suggesting that endothelial Notch1 is critical for embryonic vascular development and viability. In contrast, Notch4−/− mice are viable without any observable vascular defects. However, Notch1−/−/Notch4−/− mice exhibit a more severe vascular phenotype than Notch1−/− mice, suggesting that Notch1 and Notch4 may have overlapping roles in vascular remodeling and morphogenesis during development. The role of Notch1 and Notch4 signaling in postnatal vascular angiogenesis and remodeling remains however to be determined.

In addition to cell death, we demonstrate that Notch signaling regulates EC dysfunction that plays a major role in many chronic inflammatory disorders as in TA.

In conclusion, we show here that Notch4 downregulation during TA and in ECs is associated with a global decrease in Notch activity. Notch4 downregulation functionally results in EC dysfunction suggesting that Notch4 and basal Notch activity are required to maintain EC quiescence and for optimal survival and repair in response to injury. Overall, we also show that regulation of Notch4 alone had substantial effects, suggesting that it is one of the main Notch receptor in vascular endothelium.

Acknowledgments

The authors thank Philippe Hulin and the “confocal microscopy and cellular imaging platform” of IFR26 for time lapse study.

Sources of Funding

This work was supported by “Xenome”, a European Commission-funded Integrated Project, Life Sciences, Genomics and Biotechnology for Health LSHB-CT-2006-037377, and by fellowships from La Societe Francophone de Transplantation, La Societe de Nephrologie et La Fondation Pregreff, T.Q. was supported by a grant from la Fondation pour la Recherche Medicale.

Disclosures

None.

References

Quillard et al. Altered Notch4 Elicits EC Activation and Apoptosis


Impaired Notch4 Activity Elicits Endothelial Cell Activation and Apoptosis: Implication for Transplant Arteriosclerosis
T. Quillard, S. Coupel, F. Coulon, J. Fitau, M. Chatelais, M.C. Cuturi, E. Chiffoleau and B. Charreau

Arterioscler Thromb Vasc Biol. 2008;28:2258-2265; originally published online September 18, 2008;
doi: 10.1161/ATVBAHA.108.174995

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/28/12/2258

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2008/09/19/ATVBAHA.108.174995.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Methods

Animal model of Transplant Arteriosclerosis

Rats heterotopic cardiac allografts were performed as previously described with fully MHC mismatched LEW.1W (RT1u) or syngeneic LEW.1A (RT1a) as transplant donors and LEW.1A as recipients (Centre d’Elevage Janvier, Genest-Saint-Isle, France) 1. Recipients underwent either a donor-specific blood transfusion (DST) 7 and 14 days before transplantation (n=4) or a 20-day course of the deoxyspergualin analog LF015-095 (n=4). At day 100 post-transplantation, clear-cut histological signs of transplant arteriosclerosis (TA) were found in DST-treated animals whereas LF015-095-treated animals tolerated the graft without any signs of TA 1, 2. Graft survival was evaluated by palpation through the abdominal wall. Studies described here have been performed in accordance with the institutional guidelines of the INSERM. Messenger RNA and proteins extraction and Immuno-histological stainings were performed on entire grafts 100 days post-transplantation.

Rat cellular subsets were isolated from LEWIS rat as following. EC of LEW.1A origin were isolated and cultured as previously described 3. Total CD103+ DCs were selected and stimulated with CD40L for 6h as previously described 4. B cells (TCR- CD4- CD45R+) and NK cells (TCR- CMHIIhigh- CD8+/CD161a+high) were respectively purified from mononuclear spleen cells by FACSria flow cytometer (Bd Bioscience, Franklin Lakes, NJ, USA). The resultant purity of sorted cells was >95%. NK cells were activated with PMA (10 ng/ml) and ionomycin (1µg/ml) for 5h. T cells (His42- OX42-) were obtained after negative selection with anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway). LT were stimulated with anti-CD3 (0.75µg/mL) and with addition of soluble anti-CD28 (0.6µg/mL). Purity was routinely >95%. NR8383 Macrophages cell line was activated with LPS (1µg/ml) for 24h (Sigma–Aldrich).
Cell culture and reagents

Primary EC cultures from human artery (HAEC) and umbilical vein (HUVEC) were isolated and grown in endothelial basal growth medium (ECBM, Promocell, Heidelberg, Germany) supplemented with 10% fetal calf serum (FCS), 0.004ml/ml EC growth supplement/heparin, 0.1 ng/mL human epidermal growth factor, 1ng/mL human basic fibroblast growth factor, 1μg/mL hydrocortisone, 50μg/mL gentamicin, and 50ng/mL amphotericin B (Promocell, Heidelberg, Germany), 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin (Sigma-Aldrich). Confluent EC monolayers were deprived for 6 to 12h in ECBM with 2% FCS, 2mM L-glutamine, 100U/ml penicillin, 0.1mg/ml streptomycin before incubated with Human Recombinant TNF (100U/ml, provided by Professor P. Neuman, BASF, BASF Ludwigshafen, Germany), TGFβ or IL10 (2ng/ml) (R&D Systems, Abingdon, UK). For inhibition experiments, cells were pretreated for 1h with PDTC (pyrrolidine dithiocarbamate, 100μM) or NAC (N-Acetyl-Cysteine, 10mM) (Sigma–Aldrich , Saint Quentin Fallavier, France) before TNF treatment for 24h.

Plasmids, siRNA transfection and CBF1-luciferase reporter assay

The luciferase reporter plasmid that contains 4 copies of a binding site for CBF1 (CBF1-Luc) was a kind gift from Dr. Diane Hayward (Johns Hopkins University, Baltimore, Maryland, USA) 5. Cells were co-transfected with the N2IC/MSCV plasmid encoding Notch2 NICD kindly provided by Dr Christopher A. Klug CA (University of Alabama at Birmingham, AL, USA) as a positive control for Notch activity 6. HUVEC were transfected using the Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) and were incubated with TNF or DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester, Sigma–Aldrich). Gene reporter activity was measured with the luciferase assay (Promega, Madison, WI, USA) and expressed as a relative luciferase activity after normalization to protein content.

For gene silencing, cells were transfected with RNAiMax lipofectamine® (Invitrogen) and siRNA targeting Notch4 (#107458, 95% knockdown, #107459, 74% knockdown), Hes1 (#114545, 73% knockdown, #114545, 78% knockdown) or a scrambled negative control (#AM4611) (Ambion,
Austin, TX, USA) at a final concentration of 10nM. Specific expression knockdown by siRNA was attested by qRT-PCR and functional assays were assessed 48h post-transfection.

**Quantitative real-time PCR**

RNA was isolated using Trizol reagent (Invitrogen) and treated with Turbo DNase® (Ambion) before reverse transcription (RT). Quantitative PCR were performed using the ABI PRISM 7700 and 7900 sequence detection application program (PE Applied Biosystems, Foster City, CA, USA). For quantification, duplicates were normalized by the concomitant quantification of hypoxanthine-guanine phosphoribosyl transferase (HPRT) and by an independent Rat or Human referent sample. Relative expression was calculated according to the 2-ΔΔCt method, as previously described 7. Custom primers for Hes1 (5’-AGAGGCGGCTAAGGTTGGTGTGTTTTTGGGAT-3’, 5’-GAGAGGTGGGTTGGGGAGTT-3’) and HPRT (5’-CCTTTGGTCAGCATGACACAAACAGTGA-3’, 5’-TTCACTGATTGACACAAACAGTGA-3’) were obtained from MWG (High Point, NC, USA) and used for qRT-PCR analysis. Primers and probes for the following genes were purchased from Applied Biosystems: Notch1 (Hs00413187_m1, Rn01758641_g1), Notch2 (Hs00225747_m1, Rn00577522_m1), Notch3 (Rn00571731_m1), Notch4 (Hs00270200_m1; Rn01525737_g1), VCAM-1 (Rn00563627_m1), TGFβ (Rn00821748_g1), TNF (Rn99999017_m1), IL10 (Rn01483989_m1) and HPRT (H99999909_m1; Rn01527838_g1)

**Western blot analysis**

Cells were lysed on ice in 20mmol/L Tris-HCl (pH7.4), 137mmol/L NaCl, 0.05% Triton X-100, 1 mmol/L phenylmethlysulfonyl fluoride supplemented with Protease Inhibitors Cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA (PIC, Sigma–Aldrich). Cell lysates were resolved by SDS-PAGE (7.5%–10%) and subjected to Western immunoblot with antibodies for Notch4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Notch1 (Millipore, Billerica, MA, USA) and VCAM-1 (Santa Cruz Biotechnology), anti-GAPDH, anti-tubulin (Oncogene, MERCK EuroLab, Val de Fontenay, France) and secondary horseradish peroxidase-labeled (CST, St Quentin-en-Yveline, France; Serotec, Martinsried, Germany).
Antibody-bound proteins were detected using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Results shown are representative of at least 3 independent experiments.

**Immunohisto-Chemistry**

Rat tissues (hearts) were collected, embedded in Optimal Cutting Temperature compound (OCT Compound; Tissue Tek, Miles Laboratories, Elkhart, IN, USA), immediately snap frozen in liquid nitrogen, and then stored at –80°C until use. Cryostat sections from entire grafts of 5µm were thawed, fixed in acetone (10 minutes at room temperature), and incubated overnight at 4°C with monoclonal mouse anti-Notch4 or anti-PECAM-1 (an endothelial cell marker, 5µg/ml) mAb purchased from CST and Serotec, respectively. Sections were then labeled using a three-step indirect immuno-peroxidase technique with a biotin-conjugated anti-mouse Ab (Jackson ImmunoResearch, West Grove, PA, USA), HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA, USA), visualized with VIP substrate (Vector Laboratories), and counterstained by incubation with hematoxylin and lithium chloride. Controls were performed using the blocking solution (PBS, 5% BSA, 5% normal donkey serum) in place of the primary antibodies. Sections were mounted in ProLong AntiFade Kit (Molecular Probes) and analyzed by microscopy.

**Flow Cytometry**

After transfection, HAEC were harvested and incubated with anti-human VCAM-1 (10µg/ml, R&D systems) then with a FITC-labeled polyclonal goat anti-mouse IgG F(ab’)2 antibody (Jackson Laboratories, West Grove, PA). Fluorescent labeling was measured on 10,000 cells/sample using a FACScalibur® (BD Biosciences) and analyzed with FlowJo® software (Tree Star, Inc., Ashland, OR, USA).

**Scratch Assay & Time Lapse**

Confluent cell layers were scratched with a 20µl micro-pipette tip, washed twice with PBS to remove cell debris and subjected for time-lapse video microscopy for 24h in complete culture medium. Phase contrast videos were taken using a computer-assisted microscope (Leica DMI6000B, Leica...
Microsystèmes SAS. Rueil Malmaison) equipped with an objective lens X5 (HCX FL Plan), and a CCD camera (Coolsnap HQ2, Photometrics Roper Scientifics SAS Evry). Results of all experiments were calculated as a ratio of cell-covered areas after 24h to initial scraped areas which did not differ significantly. Image acquisition and analysis were performed by using MetaMorph software (Universal Imaging, Media, PA).

Cell Viability and Apoptosis Assays

**MTT assay** - After 48h post-transfection, cells were washed with PBS and 1ml of MTT (Methylthiazolyldiphenyl-tetrazolium bromide, 5mg/mL) was added to each well (Sigma Aldrich). After 4h at 37°C, Formazan crystals were solubilized in DMSO. OD for each condition was measured in triplicates at 570nm using a microplate reader (MRX, Dynatech laboratories, Chantilly VA, USA).

**Annexin V staining** - For apoptosis detection, EC were harvested, washed and resuspended in Binding Buffer (BD Bioscience) with annexinV-APC for 15min. Fluorescence was measured on 10,000 cells/sample using a FACScalibur® (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo® software (Tree Star, Inc.). Results shown are representative of at least 3 independent experiments.

**Statistics**

Data are represented as means±SE for replicates experiments. Statistical analysis was performed with Graphpad Prism® Software (Graphpad Software, San Diego, CA) by the parametric analysis of variance test. p<0.05 was considered statistically significant.

Supplemental References


**Notch4 expression in immune cellular subsets.** Resting and activated immune cellular subsets from LEW rat were subjected to qRT-PCR analysis for Notch4 mRNA and normalized by HPRT quantification. T Cells were stimulated with anti-CD3 and -CD28 mAb for 24h, NK cells were stimulated with PMA and ionomycin for 5h, Macrophages were stimulated with LPS for 24h and dendritic cells (DC) with CD40L for 6h. Data shown are means ±SE from 3 determinations.
NFκB-dependent regulation of Notch4 in EC. HAEC were preincubated with PDTC (100µM) or NAC (10mM) for 1 h before a 24 h-treatment with TNF (100 U/ml). Quantitative RT-PCR analysis for Notch4 mRNA expression (A) and a representative analysis of Notch and VCAM-1 protein expression by Western blotting (B) were performed. (C) Notch activity was further analyzed by quantification of Hes1 transcript level using qRT-PCR in HAEC. (D) HUVEC were transfected with a luciferase reporter plasmid CBF1-Luc before treatment. Co-transfection of both CBF1-Luc and the N2NICD plasmids was used as a positive control for Notch activity. Results shown are means of 4 independent experiments and are expressed as relative luciferase activity (in arbitrary units). (*p < 0.05 versus respective control)
Supplemental Figure III

**Specificity of Notch4 knockdown by siRNA.** HAEC were transiently transfected with no siRNA (medium), a scramble siRNA (scramble) or two different siRNAs targeting Notch4 (siNotch4#1 and #2) (10nM each) using RNAiMax lipofectamine®. Notch1, Notch2 and Notch4 mRNA was analyzed by qRT-PCR 48h post transfection and normalized to HPRT level. Results shown are means±SE from 3 independent experiments expressed as percentages of controls (medium), *p < 0.05 versus controls.