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The Notch Pathway Attenuates Interleukin 1β (IL1β)-mediated Induction of Adenylyl Cyclase 8 (AC8) Expression during Vascular Smooth Muscle Cell (VSMC) Trans-differentiation®(*)

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Background: Adenylyl cyclase 8 (AC8) expression is associated with pathological vascular smooth muscle cell (VSMC) trans-differentiation.

Results: The Notch-HRT1/3 pathway inhibits IL1β up-regulation of AC8 in VSMCs.

Conclusion: Notch3 pathway down-regulation (in the inflammatory context of pathological vascular remodeling) is a major event for sparking AC8 expression.

Significance: AC8 ranked as a marker of trans-differentiated VSMCs contributes to understanding the mechanisms involved in atherosclerosis and postangioplasty restenosis.

Vascular smooth muscle cell (VSMC) trans-differentiation, or their switch from a contractile/quiescent to a secretory/inflammatory/migratory state, is known to play an important role in pathological vascular remodeling including atherosclerosis and postangioplasty restenosis. Several reports have established the Notch pathway as tightly regulating VSMC response to various stress factors through growth, migration, apoptosis, and de-differentiation. More recently, we showed that alterations of the Notch pathway also govern VSMC acquisition of the inflammatory state, one of the major events accelerating atherosclerosis. We also evidenced that the inflammatory context of atherosclerosis triggers a de novo expression of adenylyl cyclase isoform 8 (AC8), associated with the properties developed by trans-differentiated VSMCs. As an initial approach to understanding the regulation of AC8 expression, we examined the role of the Notch pathway. Here we show that inhibiting the Notch pathway enhances the effect of IL1β on AC8 expression, amplifies its deleterious effects on the VSMC trans-differentiated phenotype, and decreases Notch target genes Hrt1 and Hrt3. Conversely, Notch activation resulted in blocking AC8 expression and up-regulated Hrt1 and Hrt3 expression. Furthermore, over-expressing Hrt1 and Hrt3 significantly decreased IL1β-induced AC8 expression. In agreement with these in vitro findings, the in vivo rat carotid balloon-injury model of restenosis evidenced that AC8 de novo expression coincided with down-regulation of the Notch3 pathway. These results, demonstrating that the Notch pathway attenuates IL1β-mediated AC8 up-regulation in trans-differentiated VSMCs, suggest that AC8 expression, besides being induced by the proinflammatory cytokine IL1β, is also dependent on down-regulation of the Notch pathway occurring in an inflammatory context.

Atherosclerosis, a chronic disease of the arterial wall, and related cardiovascular incidents, are the number one causes of death throughout the world (World Health Organization). Trans-differentiation of vascular smooth muscle cells (VSMCs), or their switch from a contractile/quiescent to a secretory/proliferative state, is known to play an important role in the development of atherosclerotic lesion development and/or postangioplasty neointimal formation. Therefore, many efforts are being made to determine the molecular mechanisms that are engaged in this process.

Over the past decade, several studies identified Notch as a critical player in controlling cell trans-differentiation by regulating intercellular communications and direct cell fate decisions (1, 2). In mammals, four Notch proteins have been discovered (Notch1–4), each being a large single-pass type 1 transmembrane receptor. Currently, five membrane-bound Notch ligands are known (Delta-like 1, 3, 4, and Jagged 1–2). Upon ligand binding, proteolytic cleavages (which involve extracellular proteases of ADAM/TACE, a disintegrin and metalloprotease/tumor necrosis factor α converting enzyme,}

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3 The abbreviations used are: VSMC, vascular smooth muscle cell; AC, adenylyl cyclase; ADAM, a disintegrin and metalloproteinase; CD31, cluster of differentiation 31 also known as platelet endothelial cell adhesion molecule; DAPT, N-(N-[3,5-difluorophenacetyl]-L-alanyl)-S-phenylglycine t-butyl ester; HES, hairy and enhancer of split; HRT, hairy-related transcription factors; NF-κB, nuclear factor-κB; NICD, Notch intracellular domain; RBP-Jκ, recombinant signal-binding protein 1 for Jκ; RBP-Jκ-DN, RBP-Jκ dominant-negative; SM22, smooth muscle 22α; ICD, intracellular domain; EGFP, enhanced green fluorescent protein.
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EXPERIMENTAL PROCEDURES

Ethical Approval—All procedures were performed in accordance with European Community standards on the care and use of laboratory animals approved by the ethics committee for animal experimentation (Ile de France-Paris-Committee 3, Authorization 4270).

Ballooning Injury of the Rat Carotid Artery—Adult male Wistar rats (Charles River, MA) weighing 400 g were anesthetized with pentobarbital (50 mg/kg intraperitoneally). Heparin (35 IU) was administered systemically by intraperitoneal injection. The left external carotid artery was injured using a 2F Fogarty embolectomy catheter (Baxter Healthcare Corp.) introduced into the common carotid artery through the external carotid, inflated to 2 atmospheres and withdrawn 3 times. The catheter was removed and the incision hole was ligated. Perfusion was restored in the common carotid and the neck incision was closed using 4-0 silk sutures. Carotids were collected 7 and 14 days postinjury, (after intraperitoneal injection of a lethal dose of pentobarbital) included in cryomatrix (OCT), and frozen at −80 °C. 12-μm Cross-sections were made from the entire length of the carotid and used for immunohistochemistry analysis.

Immunohistochemistry and Confocal Analysis—Tissue sections were fixed with acetone for 10 min at −20 °C. After blocking the nonspecific binding sites with normal goat serum, the tissue sections were incubated 1 h at 37 °C with rabbit anti-AC8 (Santa Cruz Biotechnology), rabbit anti-Notch3 (Abcam), mouse anti-PECAM1 (Chemicon International); rabbit anti-SM22 (Abcam); followed by incubation with the secondary antibody, Dylight 549 donkey anti-rabbit (Jackson ImmunoResearch), and Alexa 546 (Abcam). Tissue sections were examined with a Leica TCS4D confocal scanning laser microscope using Plan Apochromat objectives. Stacks of images were collected every 0.5 μm along the z axis. All settings were kept constant to allow comparison.

Immunocytochemistry—VSMCs were seeded directly onto glass coverslips placed in 12-well culture plates. After treatment (indicated in figure legends), cells were washed twice with PBS and fixed during 20 min at room temperature in 4% paraformaldehyde (neutralized with 50 mM of NH4Cl for 10 min), permeabilized in PBS + 0.2% Triton X-100, and incubated for 1 h in PBS + 10% FCS with mouse monoclonal α-actin antibody (DAKO) and rabbit polyclonal SM22 antibody (Abcam). Incubation with Dylight 488 goat anti-mouse secondary antibody and Dylight 546 donkey anti-rabbit secondary antibody was performed during 1 h followed by staining of cell nuclei with DAPI for 5 min. Coverslips were mounted with Dako fluorescent mounting medium (Dako, Carpinteria, CA). Cells were examined by confocal microscopy.

Cell Culture and Treatment Conditions—All procedures were performed in accordance with the European Community Standards on the care and use of laboratory animals and conform to NIH Guidelines. Aortas were isolated from anesthetized 5-week-old male Wistar rats (180 g). After removing the fat tissue the aortas were subjected to enzymatic digestion at 37 °C under gentle shaking in Dulbecco’s modified Eagle’s medium (DMEM) containing glucose (4.5 g/liter) and a mixture of type I collagenase 45 min. The VSMC-rich media was sepa-
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dated from the adventitial layer with forceps, dilacerated in small rings, and re-incubated in the same mixture with elastase (1.5 g/liter) for 30–45 min to dissociate VSMCs. Cells were then collected by centrifugation (3 min at 500 x g) and re-suspended in complete medium containing DMEM supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml), grown on calf skin type 1 collagen-coated dishes at 37 °C in a 5% CO2 environment. VSMCs were subcultured every 5 days in the same medium. All experiments were performed on cells at passages ranging from 2 to 6. Immunochemistry experiments visualizing α-actin show that among rat aorta vascular cell types, plated cells always display a well organized network of actin stress fibers in control untreated conditions. Twenty-four hours before any treatment, confluent cells were starved and experiments were continued in serum-free culture medium. Control cells were harvested at the same time as treated cells to prevent any effect of starvation. All interleukin-1β (IL1β) incubations were performed with human IL1β (PeproTech) dissolved in 2.5 µM BSA (bovine serum albumin, Sigma). Therefore, all incubations were performed in the presence of 2.5 µM BSA. When treated with Jagged1-Fc ((rat jagged 1, Ser32–Asp1068 (Gly57–Arg59del), D63T, R64L, and Val-Arg-P6Yins before Lys69) IEG-MRD-Human IgG1 (Pro100–Lys130)-His, tag; truncated at its C terminus and lacks the transmembrane as well as the intracellular domain), the ligand was clustered with goat anti-human Fc antibodies for 1 h prior to IL1β treatment. N-[N-(3,5-Difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) was dissolved in dimethyl sulfoxide (Sigma). The final concentration of dimethyl sulfoxide (0.01%) did not have any effect (as compared with control untreated cells) on the measured parameters (data not shown).

**Adenoviral Infection**—Rat VSMCs were infected with adenovirus expressing Ad-βGal, encoding β-galactosidase-GFP under the CMV promoter (19) Notch1 IC-IRE-EGFP or Notch3 IC-IRE-EGFP (20) (Ad-βGal, Ad-Notch1 IC, Ad-Notch3 IC, respectively) at a multiplicity of infection of 10 in complete medium for 36 h prior to serum starvation and subsequent treatment as indicated in the figure legends. Infection efficiency was controlled by counting GFP versus non-GFP positive cells.

**RT-PCR**—Total RNA of rat VSMCs and rat carotid arteries was extracted from VSMCs using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After annealing oligo(dT) (1 µM) to template RNAs (0.5 µg) at 70 °C for 5 min, primer extension was initiated by adding the RT-Moloney murine leukemia virus enzyme plus 0.5 mM dNTP, 1 unit of RNAsin, and 10 µM dithiothreitol, and carried out for 45 min at 37 °C. Quantitative PCR was performed using the LightCycler LC480 (Roche Diagnostics). The PCR mixture included 5 µl of each reverse transcriptase (diluted 1:25) and 300 nM of each primer in 1X LightCycler DNA SYBR Green 1 Master Mix. Specific primers for complementary DNA (cDNA) were chosen with the LightCycler Probe Design2 program according to European Molecular Biology Laboratory accession numbers. The forward and reverse primers used to selectively amplify the cDNA encoding rat hypoxanthine phosphoribosyltransferase, AC3, AC6, AC8, HRT1–3, HES1, HES5, Notch1, Notch3, RBP-Jκ, α-actin, and SM22 are presented in Table 1. The PCRs were performed using the following thermal settings: denaturation and enzyme activation at 95 °C for 8 min, with cycling at 95 °C for 10 s, 64 °C for 10 s, and 72 °C for 8 s. Amplification was followed up online, and the PCRs were stopped after the logarithmic phase. Melting curve analyses were performed after PCR to check the reaction specificity. Controls and water blanks were included in each run; these were negative in all cases. Real-time quantitative PCR data were represented by the amount of each target messenger RNA (mRNA) relative to the amount of mRNA for a housekeeping reference gene, here, hypoxanthine phosphoribosyltransferase, estimated in the logarithmic phase of the PCR. Serial dilutions of reverse transcription products were used to determine the fit coefficients of the relative standard curve. When the PCR efficiencies of the targets were similar, individual cultures could be compared. If not, an internal calibrator was used to compare individual cultures. Results are expressed as fold over control (untreated) cells.

**Transfection**—Smooth muscle cells were transfected by electroporation in the Amaza electroporation device using the D-33 program. Briefly, 1 million cells were re-suspended in 100 µl of Amaza electroporation transfection solution and 4 µg of each vector (RBP-Jκ-DN, 1κBα(32–36), HRT1-myc, HRT2-myc, HRT3-myc, 4 mg) were added. After electroporation, transfected cells were plated in two wells of a six-well plate, each containing 1 ml of cell culture medium. 24 h after plasmid transfection, the cells were starved overnight in serum-free medium then treated as indicated in the figure legends

**Protein Extraction and Western Blot Analysis**—Cells were washed with ice-cold phosphate-buffered saline and re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1...
mm EDTA, 1% Triton, 1% sodium deoxycholate) plus Complete protease inhibitor mixture (Roche Diagnostics) for 10 min on ice. The lysate was centrifuged at 13,000 × g for 10 min at 4 °C. Cell lysates (20–40 μg) were analyzed by SDS-PAGE on a 12% resolving gel followed by transfer to nitrocellulose membranes (NuPAGE system). The free binding sites on membranes were blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and 5% fat-free milk for 1 h at room temperature. The membranes were then incubated overnight with primary antibody c-myc (Santa Cruz) in TBST with 5% fat-free milk for 1 h at room temperature. The membranes were then washed in TBST and incubated with horseradish peroxidase-conjugated secondary antibody (P.A.R.I.S. Ltd., France) for 1 h at room temperature followed by repeated washes with TBST. Signals were detected with the ECL detection system and exposed to Fujiﬁlm LAS-300 (Fujifim Medical Systems).

**Flow Cytometry**—To detect Jagged1-Fc binding, the cells were re-suspended in PBS with 1% BSA and treated for 1 h with 1 μg/ml of Jagged1-Fc at 4 °C. Jagged1 binding was revealed by an anti-human Fc goat polyclonal antibody followed by a secondary 549 Dylight donkey anti-goat antibody (Jackson ImmunoResearch). Endogenous Jagged1 expression was examined as follows: VSMCs were starved for 24 h and treated with or without 1.5 ng/ml of IL1β in serum-free culture medium for 48 h. Subsequently the cells were re-suspended in phosphate-buffered saline (PBS) containing 1% BSA. Cells were stained with a mouse anti-Jagged1 polyclonal antibody (Santa Cruz Biotechnology) followed by 488 Dylight goat anti-mouse secondary antibody (1/400 Jackson ImmunoResearch); Delta was detected with an anti-Delta directly coupled to APC (allophycocyanine; Biolegend). In all cases, stained cells were washed with PBS containing 1% BSA and
analyzed immediately on a MoFlow XL4 cytometer (Beckman-Coulter) with Submitt Software.

Statistical Analysis—Data are reported as mean ± S.E. The numbers of independent experiments are reported in the figure legends. One-way analysis of variance Tukey-Kramer Multiple Comparisons Test was used to compare the mean values between groups using GraphPad InStat (GraphPad Software, San Diego, CA).

RESULTS

Notch 3 Expression Opposes AC8 Expression—IL1β induces the trans-differentiation of VSMCs and stimulates the expression of AC8 (15). As an initial approach to determine whether there is any correlation between AC8 and Notch expressions in VSMCs, we analyzed their transcript expressions when treated with various concentrations of IL1β. As shown in Fig. 1A, IL1β-induced AC8 expression and Notch3 down-regulation are dose-dependent. Because AC8 transcript induction displayed an EC₅₀ value of ~1.5 ng/ml, we chose to treat VSMCs with this concentration of IL1β (which was used for all the in vitro experiments reported in this article). We next measured the kinetics of AC8 and Notch3 receptor mRNA expression over 48 h of IL1β treatment. The analysis of the kinetics revealed that AC8 mRNA expression was significant after 16 h of treatment (compared with untreated cells; see the legend to Fig. 1 for details), whereas a strong (~60%) down-regulation of Notch3 was evident as soon as 6 h (Fig. 1B). Notch1 mRNA levels, although displaying a tendency of inhibition at 16 h, did not change significantly (data not shown); similar results were obtained for Notch2 (data not shown). Altogether, this data indicates that IL1β-induced Notch3 inhibition preceded AC8 up-regulation.

Inhibition of the Notch Pathway Reinforces the Effect of IL1β on AC8 Expression—To determine the involvement of the Notch signaling pathway in AC8 expression, we coincubated VSMCs with IL1β (1.5 ng/ml) and/or DAPT (referred to as D), a potent inhibitor of the γ-secretase complex activity, for 48 h. (The γ-secretase complex generates the intracellular active domain of Notch, as mentioned under the Introduction.) As illustrated in Fig. 2A, Notch inhibition using DAPT (2.5 μM) doubled the effect of IL1β on AC8 mRNA induction; DAPT alone had no effect on AC8 transcript expression when compared with vehicle-treated (referred to as control, Ctl) cells. DAPT treatment did not have any effect on AC3 and AC6, which are two main isoforms of adenylyl cyclases present in VSMCs, although AC3 transcripts were decreased in IL1β-treated VSMCs (Fig. 2B). Lower (0.5 μM) or higher (10 μM) concentrations of DAPT provided similar results (data not shown). Notch inhibition by DAPT treatment was confirmed by attributing its inhibitory effect on Notch target genes expression (HRT1–3, HES1, and HESS) (Fig. 2C).

Apart from generating NICD, the γ-secretase complex is also responsible for intramembrane processing of over 60 substrates (21). Thus, to confirm that inhibition of the Notch signaling pathway increases IL1β-induced AC8 expression, we used the soluble human recombinant Jagged1-Fc fusion protein, reported to have an antagonistic effect on Notch signaling (22, 23) and assayed AC8 transcript expression. The main reason for choosing Jagged1 rather than Delta-like Notch ligands is that Jagged1-but not Delta-like 4-induced Notch signaling regulates VSMC differentiation (24). Jagged1-Fc binding efficiency was assessed by flow cytometry using an anti-Fc antibody on Jagged1-Fc-treated VSMC (Fig. 3A, left panel). As shown in Fig. 3A, right panel, Jagged1-Fc (1 μg/ml) treatment doubled the effect of IL1β on AC8 mRNA expression. Similar results were obtained when using a concentration of 2.5 μg/ml of Jagged1-Fc (data not shown). As expected for an antagonist, this recombinant protein significantly decreased the expression of Notch targets genes, HRT1 and -3 (Fig. 3B). Although not significant, HRT2 expression also tended to decrease, whereas HES1 and HESS were unaffected.

Once in the nucleus, NICD interacts with transcriptional repressor RBP-Jκ (recombinant signal-binding protein 1 for J-κ, also known as C promoter binding factor 1 suppressor of
hairless, Lag-1), which results in activating Notch-RBP-J target genes (3). As evidenced in Fig. 3C, left panel, overexpressing RBP-JΔx dominant-negative (RBP-JΔx-DN, shown by real time PCR in Fig. 3C, right panel) increased the levels of IL1β-induced AC8 expression, similar to DAPT and Jagged1-Fc treatment (Figs. 2A and 3A, right panel). Of note, RBP-J-DN associates with Notch ICD but lacks its DNA binding capability, therefore altering the transcription of Notch target genes in response to Notch activation (25, 26).

**Notch Pathway Inhibition Amplifies the Deleterious Effects of IL1β on VSMC Phenotype.—**To examine effects of the Notch pathway inhibition on VSMC morphology and trans-differentiation, we inhibited the Notch pathway with DAPT or Jagged1-Fc and evaluated the expression of α-actin and SM22, both markers of differentiated, contractile VSMCs. As demonstrated by RT quantitative PCR analysis, α-actin and SM22 transcripts were decreased when VSMCs were treated with IL1β (1.5 ng/ml for 48 h). This down-regulation was further enhanced when Notch was inhibited whether using DAPT (Fig. 4A) or Jagged1-Fc (Fig. 4C). As visualized by immunostaining in Fig. 4, B and D, the intensity and number of cells positive for α-actin and SM22 labeling were much lower in cells treated with Notch inhibitors (DAPT or Jagged1-Fc) and IL1β versus IL1β alone after 5 days of treatment, demonstrating that Notch
inhibition exacerbates IL1βs effect on VSMC trans-differentiation. When observing cell morphology at a higher magnification (×63), α-Actin and SM22 networks are less dense, disorganized, and display a tangled arrangement in IL1β + DAPT or Jagged1-Fc-treated cells that still expressed these markers (supplemental Fig. S4). Altogether these experiments clearly dem-
onstrate that morphology and differentiation markers (α-actin and SM22) are drastically modified along with AC8 mRNA potentiation (Figs. 2A and 3A, right panel) when Notch is inhibited in IL1β-treated cells.

**Notch3-ICD and/or Notch1-ICD Prevent the Effects of IL1β on AC8 Expression through HRT1 and/or HRT3—** We next evaluated the effects of Notch activation on IL1β-induced AC8 expression. Thus, we infected our cell model with an adenovirus expressing either the Notch3 or the Notch1 constitutively active intracellular domains (Notch1-ICD-IRES-EGFP or Notch3-ICD-IRES-EGFP). An adenovirus expressing β-gal was used as a control. Infection efficiency (estimated by counting GFP positive cells) was around 80% in all conditions and maintained upon IL1β treatment (data not shown). As evidenced in Fig. 5A, left panel, Notch1-ICD and Notch3-ICD blocked the induction from IL1β of AC8 by 70%. Both Notch1-ICD and Notch3-ICD induced significant up-regulation of the Notch downstream effector transcripts HRT1 and HRT3 but not HRT2, HES1, and HESS (Fig. 5B). Although N1-ICD had no effect on AC3 and AC6 expression whether in control or IL1β-treated cells, N3-ICD infection increased the expression of both of these cyclases (supplemental Fig. S1). Of note: IL1β-induced AC3 down-regulation (Fig. 2B) is maintained in N3-ICD-infected VSMCs (supplemental Fig. S1). This set of experiments demonstrates that Notch1 and -3 activities prevent the expression of AC8 induced by IL1β.

Regarding the systematic modifications of HRT1 and -3 gene expressions when the Notch pathway is modulated (Figs. 2C, 3B, and 5B) we next addressed the hypothesis of whether they contribute to repressing AC8 expression. To accomplish this, we overexpressed HRT1, HRT2, and HRT3 in IL1β-treated rat VSMCs and analyzed the effects on AC8 expression. Transfection efficiency was controlled by Western blot with an anti-c-myc because HRTs constructs are c-myc tagged (Fig. 6B). As shown in Fig. 6A, HRT1 and HRT3 overexpression, in contrast to HRT2, significantly decreased AC8 mRNA transcript expression. Collectively, these results suggest that in cultured VSMCs Notch1 and -3 intracellular active domains prevent the ability of IL1β to induce AC8 expression through enhancing the expression of transcriptional repressors HRT1 and/or HRT3.

**FIGURE 5. Overexpression of Notch1 and Notch3 ICD prevent IL1β-induced AC8 gene expression.** Rat VSMCs were infected with adenoviruses (Ad) containing β-gal-EGFP, Notch1-ICD-EGFP, or Notch3-ICD-EGFP for 36 h. Cells were starved in serum-free medium for 24 h, then treated with 1.5 ng/ml of IL1β for 24 h. Total RNA was extracted and AC8 (A, left panel) and Notch target genes HRT1, HRT2, HRT3, HES1, and HESS. B, gene expression levels were analyzed using real time PCR. Results are expressed as a fold-induction of the mRNA level of nontreated cells and represent the mean ± S.E. of 5 independent experiments. *, p < 0.05; **, p < 0.01 (versus control); #, p < 0.05; ##, p < 0.01 (between IL1β-treated cells). A, right panel, Ad-β-gal-EGFP, Ad-Notch1-ICD-EGFP, and Ad-Notch3-ICD-EGFP protein expression of VSMCs after 36 h of infection (×20).
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As an initial approach to evaluate the concordance of this in vitro finding with in vivo VSMC remodeling toward the inflammatory/migratory/proliferative state, we used the rat carotid balloon injury model of restenosis (Fig. 7). The differentiation status of medial cells as well as their identity was attested by SM22 labeling (Fig. 7A). Efficiency of the balloon injury was attested by post-intervention endothelial denudation, visualized here with altered CD31 expression (endothelial cell marker). Immunofluorescence staining revealed the presence of Notch3 in medial/contractile VSMCs of control non-injured vessels, whereas AC8 expression was not detectable. At day 7 post-injury, expression of Notch3 and SM22, a marker of the contractile phenotype, were no longer detectable in medial or neointimal VSMCs, whereas AC8 expression was up-regulated. Fourteen days postinjury, Notch3 expression was restored in the neointima, but also among the (medial) elastin fibers. A similar tendency was observed for SM22 expression although its expression still seemed low at this time. This was consistent with the completion of neointimal formation usually occurring between 14 and 21 days postinjury when neointimal as well as medial VSMCs regain a differentiated state (15). In accordance with the in vitro findings mentioned above, AC8 expression opposed that of Notch3. In particular, one might notice that the expression of AC8 was absent in medial/differentiated VSMCs of non-injured vessels, but was highly present in the media and neointima 7 days after balloon injury and disappeared 14 days postintervention.

These observations were confirmed by real-time RT-PCR analysis of AC8 and Notch3 mRNA levels in post-balloon-injured rat carotids (analyzed from 2 to 30 days) (Fig. 7B). Indeed, Notch3 expression dramatically decreases 2 days postinjury prior to AC8 expression, which was maximal at day 4. AC8 up-regulation was maintained until 10 days postinjury and returned to basal levels at 30 days postinjury; simultaneously, relative expression of Notch3 was progressively restored during this same period. Furthermore, the expression of Notch ligands Delta1 and Jagged1, and target genes HRT1, HRT2, HRT3, and Hes1 were also down-regulated in rat carotid arteries 2 days postinjury, (supplemental Fig. S3). Therefore, whether in vitro or in vivo, the inhibition of the Notch3 signaling pathway precedes AC8 expression up-regulation and the two are reciprocally related.

Taken together, this data demonstrated that AC8 expression is dependent on Notch pathway down-regulation in inflammatory, trans-differentiating VSMCs and strongly support that AC8 gene silencing occurring in a noninflammatory context potentially relies on Notch-induced expression of HRT1 and/or HRT3 transcriptional repressors.

DISCUSSION

The expansion of atherosclerotic lesions and postangioplasty restenosis is due, in part, to the phenotypic transition of medial VSMC. Indeed, this transition (initiated by macrophage secretion of proinflammatory cytokines and growth factors within the intima) forces these cells to switch from a contractile/quiescent to a secretory/proliferative state (referred to as the trans-differentiation process) allowing them to migrate and proliferate toward the intima and secrete, in turn, inflammatory molecules. Therefore, identifying the molecular mechanisms/entities, which control or participate to this phenotypic switch appears crucial to advance therapeutic strategies for atherosclerosis and/or to develop new molecular target(s) of stent-eluting molecules to improve the benefit of stent implantation.

Differentiated VSMC are associated with high expressions of several specific contractile proteins including SM22 and smooth muscle α-actin (27). Our results demonstrate that Notch inhibition via DAPT or Jagged1-Fc treatment in an inflammatory context modifies the VSMC phenotype by decreasing differentiation markers α-actin and SM22 (Fig. 4). This is in agreement with previous studies showing that Notch can directly or indirectly regulate expression of VSMC differentiation markers (24, 28–30). Similar results were observed in vivo in the rat carotid balloon injury model of restenosis, where SM22 expression drastically decreases postinjury concomitantly with Notch3 down-regulation and AC8 induction (Fig. 7A).

We (10, 14, 15) and others (5) have shown the involvement of both Notch and AC8 in the regulation of vascular smooth muscle cell transition toward a trans-differentiated phenotype. Nevertheless, none of these studies have established the link between Notch signaling and AC8 up-regulation. This study highlights, for the first time, the dependence of AC8 de novo expression on the regulation of the Notch signaling pathway. The most convincing arguments in support of this are the reported potentiating effect of the recombinant Jagged1-Fc on IL1β-induced AC8 expression (Fig. 3) and the observed AC8 down-regulation when overexpressing Notch3 or Notch 1 (Fig. 5) and HRT1 or HRT3 (Fig. 6). These findings also confirm this particular AC isoform as a marker of inflammatory VSMCs trans-differentiated status. The fact that neither AC3 nor AC6 expression (which are two AC isoforms well expressed in dif-
differentiated/contractile VSMCs) were affected in the same manner as AC8, by modulating Notch signaling, reinforces this conclusion (supplemental Fig. S1).

Taking our results into account and those obtained by others, we propose the Notch3 pathway as a key regulator of AC8 expression in VSMCs even though both intracellular domains, N3-ICD and N1-ICD, efficiently block its expression. Indeed, the mRNA transcript expression analysis of balloon-injured rat carotid artery tissues evidenced that only Notch3 inhibition parallels the emergence of AC8 (Fig. 7B and supplemental Fig. 3, upper right panel); earlier reports (10, 15, 31) and our results confirmed that Notch3 down-regulation precedes AC8 expression in response to IL1β treatment (Fig. 1) and in vivo in balloon-injured rat carotid arteries (Fig. 7). Notch1 expression showed not to be significantly different as compared with non-treated (control) cells; only a slight down-regulation of Notch2 expression was found (data not shown); Notch4 is not expressed neither in differentiated nor in trans-differentiated VSMCs (32).

Even though recombinant Jagged1-Fc has been shown to bind VSMCs (Fig. 3A, left panel), the Notch isoform(s) interacting with it remain(s) to be identified. Nevertheless, several arguments strongly suggest that Jagged1 could preferentially bind Notch3. For example, Liu et al. (33) published that Jagged1 is necessary for Notch3 expression in mural cells and that Notch3 could promote its own expression; Notch3 expression is strongly down-regulated (∼70%, supplemental Fig. S2B) by Jagged1-Fc in VSMCs, whereas Notch1 expression is poorly affected (supplemental Fig. S2B); Shimizu et al. (34) reported that the purified extracellular region of Jagged1 binds Notch3 with a slightly higher affinity than Notch2 but significantly more than Notch1.

**FIGURE 7.** Notch3 and AC8 expression are reciprocally related in vivo. A, expression of AC8, Notch3, SM22, and CD31 in rat carotid arteries after balloon injury. Confocal immunofluorescence (red) of noninjured and 7- or 14-day post-balloon injury rat carotid arteries using anti-AC8, Notch3, SM22, or CD31 primary antibodies. Autofluorescence of elastin is observed in green. The images are representative of independent experiments performed on 3 to 6 rats. A, adventitia; m, media; ni, neo-intima; l, lumen; ec, endothelial cells. B, total RNA was extracted from rat carotid arteries after balloon injury at the indicated time points; AC8 and Notch3 gene expressions were analyzed using real time PCR. Results are expressed as a fold-induction of the mRNA level of noninjured arteries. Data represent the mean ± S.E. of 3–7 rats depending on the time point. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
The Notch Pathway Attenuates AC8 Expression

Regarding our results demonstrating that the Fc-bound soluble form of Notch ligand Jagged1 acts as an antagonist of the Notch pathway in VSMCs, it could be expected that the membrane-bound Jagged1 decreases IL1β-induced AC8 expression through Notch activation, maintaining their differentiated state. Consistent with this, Doi et al. (24) reported that Jagged1 signaling induces smooth muscle differentiation; results leading to similar conclusions were obtained by High et al. (35). The fact that this ligand is down-regulated in an inflammatory context (in presence of IL1β, supplemental Fig. S2A, right panel) is already in favor of this. Additional studies using the soluble versus full-length Jagged1 adenoviruses (36) should be performed to validate this hypothesis.

Notch preventing AC8 expression occurs via a RBP-Jκ-dependent, as opposed to independent, transcriptional pathway (25, 26, 37). Activation of the canonical RBP-Jκ-dependent Notch pathway leads to expression of HES and/or HRT transcriptional repressor factors. When treating VSMCs with Jagged1-Fc and/or IL1β (Fig. 3B, data not shown, and supplemental Fig. S2C), we showed that among all tested down-regulated target genes, HRT1 and HRT3 were the most sensitive for the Jagged1-Fc-induced Notch pathway inhibition. Although the Alexander et al. (31) study demonstrated HRT2 being the only Notch target gene down-regulated following IL1β treatment, HRT2 down-regulation does not seem to be involved in the AC8 increased expression. Indeed, overexpression of N3-IκD or N1-IκD blocks AC8 expression along with an increase of HRT1 and -3 (Fig. 5). Finally, overexpressing HRT1 and HRT3 significantly decreased IL1β-induced AC8 expression (Fig. 6, left panel) and the only Notch target genes to be down-regulated with comparable kinetics to Notch3 are HRT1 and HRT3 (supplemental Fig. S2C). Altogether, this demonstrates that HRT1 and/or HRT3 are involved in Jagged1-Notch-dependent AC8 regulation. Because HRT1 and HRT3 mRNA levels were significantly down-regulated in post-ballooning-injured rat carotids (Refs. 7 and 8, our data, and supplemental Fig. S3), the AC8 gene silencing in VSMC can rely on the in vivo HRT3 and/or HRT1 repressing effect. By causing decreased expression of HRT1 and HRT3 (supplemental Fig. S2C), IL1β treatment could liberate E-boxes of the AC8 promoter to allow the NF-κB-dependent transcription of this gene, because IL1β/NF-κB pathways are known to be involved in the inflammatory response of VSMC (10, 11, 37). Preventing NF-κB activation by overexpressing a mutated nonphosphorylatable/nondegradable form of IkBα (32–36), reverses the AC8 up-regulation mediated by IL1β (supplemental Fig. S5). (Of note: Notch target protein families Hes and HRT are known to bind E-box DNA sequences (CACNAG and CANNTG (4).) Because our unpublished data are in favor of the existence of two distinct tissue-specific promoter regions (one driving AC8 expression in the brain and another driving its expression in trans-differentiated VSMCs), demonstrating HRTs and NF-κB direct binding to E-boxes and NF-κB responsive elements would require the cloning and characterization of the VSMC-specific AC8 promoter.4

Compiling our in vitro and in vivo studies and according to study by Doi et al. (24) stating that Jagged-1 is the main ligand for VSMC related signaling, it is most likely that the repression of AC8 expression in differentiated VSMCs occurs through Jagged1 activation of the Notch3-HRT3 and/or HRT1 pathway. Therefore, the most relevant experiment to be performed in vivo would be to follow AC8 expression and neointimal formation in mice overexpressing HRT3 and/or HRT1 in SMC. Because the very recent paper by Wu et al. (38) shows a cause to effect relationship between the Jagged1-Notch pathway inhibition and the VSMC de/trans-differentiation leading to the increase of neointimal formation using jagged1 null mice (38), our hypothesis linking neointimal formation and AC8 expression is even more likely.

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