Inhibition of Notch signaling pathway attenuates sympathetic hyperinnervation together with the augmentation of M2 macrophages in rats post-myocardial infarction

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Yin J, Hu H, Li X, Xue M, Cheng W, Wang Y, Xuan Y, Li X, Yang N, Shi Y, Yan S. Inhibition of Notch signaling pathway attenuates sympathetic hyperinnervation together with the augmentation of M2 macrophages in rats post-myocardial infarction. Am J Physiol Cell Physiol 310: C41–C53, 2016. First published October 21, 2015; doi:10.1152/ajpcell.00163.2015.—Inflammation-dominated sympathetic sprouting adjacent to the necrotic region following myocardial infarction (MI) has been implicated in the etiology of arrhythmias resulting in sudden cardiac death; however, the mechanisms responsible remain to be elucidated. Although being a key immune mediator, the role of Notch has yet to be explored. We investigated whether Notch regulates macrophage responses to inflammation and affects cardiac sympathetic reinnervation in rats undergoing MI. MI was induced by coronary artery ligation. A high level of Notch intracellular domain was observed in the macrophages that infiltrated the infarct area at 3 days post-MI. The administration of the Notch inhibitor N-N-(3,5-difluorophenacetyl-l-alanyl)-S-phenylglycine-t-butyl ester (DAPT) (intravenously 30 min before MI and then daily until death) decreased the number of macrophages and significantly increased the M2 macrophage activation profile in the early stages and attenuated the expression of nerve growth factor (NGF). Eventually, NGF-induced sympathetic hyperinnervation was blunted, as assessed by the immunofluorescence of tyrosine hydroxylase. At 7 days post-MI, the arrhythmia score of programmed electric stimulation in the vehicle-treated infarcted rats was higher than that in rats treated with DAPT. Further deterioration in cardiac function and decreases in the plasma levels of TNF-α and IL-1β were also detected. In vitro studies revealed that LPS/IFN-γ upregulated the surface expression of NGF in M1 macrophages in a Notch-dependent manner. We concluded that Notch inhibition during the acute inflammatory response phase is associated with the downregulation of NGF, probably through a macrophage-dependent pathway, thus preventing the process of sympathetic hyperinnervation.

It is well understood that recruited macrophages and activated microglia are implicated in neuroinflammation through the induction or modulation of a broad spectrum of cellular responses, most of which are involved in the early inflammatory stage (37). CD68-immunoreactive macrophages frequently exhibit nerve growth factor (NGF) immunoreactivity in myocardial damage, supporting the idea that the immune and nervous systems are linked and confirming that macrophages are necessary for enhanced sympathetic sprouting in the peri-infarct tissue (27, 46). At the initiation of the infarction, a strong inflammatory response is initiated (42), in which macrophages have been identified as pivotal cellular effectors (33). These macrophages show a time-dependent pattern of activation types. Infiltrated and damaged cells secrete various cytokines and activate macrophages to the subtypes M1 (classically activated macrophages) or M2 (alternatively activated macrophages) (30). Myocardial infarction involves a sustained influx of macrophages. The M1 subtype dominates the inflammatory phase and shows high levels of IL-1β, IL-6, and TNF-α at the early stage, whereas the macrophages involved 4–7 days post-MI are less inflammatory and express genes associated with the prototypical M2 subtype (42). These diverse and seemingly contrasting functions position macrophage as a central cellular protagonist (1, 16). Knowledge of the range of macrophage activation states and the cues that induce polarization is far from complete, and whether therapeutically modulating the activation type of macrophages is a beneficial strategy for minimizing secondary complications after MI is unknown (4, 17).

Notch signaling is an important pattern recognition receptor that is critically involved in myeloid differentiation, inflammation, and smooth muscle cell proliferation and is implicated in diseases such as atherosclerosis, diabetes, and other metabolic diseases (32). In the canonical signaling pathway, interaction of Notch receptors with their ligands (Delta-like or Jagged) leads to the proteolytic cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD), which migrates into the nucleus. In the nucleus, NICD associates with the transcription factor recombination signal binding protein J (RBP-J, also known as CSL) and regulates transcription. Notch signaling primes macrophage polarization toward the M1 proinflammatory phenotype and heightens the inflammatory response. The Notch1-γ-secretase-RBP-J pathway has been suggested to regulate the expression of key inflammatory M1 genes including MCP1, inducible nitric oxide synthase (iNOS), Icam1, and Vcam1 (13, 47). These findings are relevant for understanding the molecular mechanisms of the Notch sys-
system that control macrophage differentiation and participate in the postinfarction process. The contributory role of Notch1 in bone marrow (BM)-derived inflammatory cells, such as macrophages, in cardiac remodeling is very important and is currently under investigation. However, little is known about the role of BM-derived inflammatory cells in the pathogenesis of neural remodeling.

Accordingly, we hypothesized that Notch activation would prime macrophage polarization toward the M1 phenotype at early stages post-MI. Then, macrophage-targeted pharmacological knockdown of NICD would support the M1→M2 phenotype switch, promoting the resolution of inflammation and eventually attenuating NGF-induced sympathetic nerve sprouting. Specifically, we investigated the expression and colocalization of markers of macrophages as well as the levels of NGF and neuroinflammatory cytokines closely related to these cells, which can promote neurotoxicity or neuroprotection in the infarcted heart tissue.

MATERIALS AND METHODS

Animals and Experimental Design

Male Sprague-Dawley rats (60–70 days postnatal, ~225 g; Vital River, Beijing, China) were used. All procedures were carried out according to approved protocols and guidelines established by the Shandong University Institutional Animal Care and Use Committee. After a 7-day acclimatization period, open-chest MI surgery was performed. Each rat was anesthetized with 3% pentobarbital sodium (30 mg/kg ip), intubated via tracheotomy, and ventilated with a small-animal ventilator (HX-3008; TME, Chengdu, China), with an adjusted rate of 30–40 breaths/min and a tidal volume set to 1.1–1.3 ml/100 g body wt. The heart was exposed via a left thoracotomy, and the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 polypolyene ligature as previously described (18). The infarction was confirmed by ST segment elevation, regional cyanosis, and wall motion abnormalities. A constant body temperature of 37°C was maintained with a heating pad. With respect to clinical importance, only rats with moderate infarct size (30–50%) were enrolled (24).

Protocol 1. For Notch signaling expression analysis, 26 of 32 rats were enrolled and randomly divided into two groups, the sham group (n = 8) and the MI group (n = 18). The rats were killed on 3 days post-MI, and the hearts were used for macrophage isolation, immunofluorescence study, and qRT-PCR.

Protocol 2. For the Notch inhibition experiment, 88 of the 115 rats were enrolled (7 were excluded due to infarct area limitation, and 20 died) and randomly assigned to the following four groups: group A, sham surgery + DMSO (n = 15); group B, sham surgery + N-N-(3,5-difluorophenacetyl-l-alanyl)-S-phenylglycine-t-butyl ester (DAPT) (n = 15); group C, ligation surgery + DAPT (MI rats received DAPT therapy) (n = 30); and group D, ligation surgery + DMSO (n = 28). DAPT (cat. no. B9592; Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO at a dose of 10 mg/kg in a final volume of 50 μl and delivered via tail vein injection 30 min before experimentation and then once per day until the end of the experiment (6, 48). DMSO, the vehicle for DAPT, was administered in the same manner in the respective vehicle groups (19). In each drug-treated group, drugs were withdrawn ~24 h before the end of the experiments to eliminate pharmacological actions (11). The rats were killed on 3 and 7 days post-MI or sham surgery because inflammatory macrophages peak at day 3 and are most pronounced within the first week and because, in rodents and dogs, sympathetic hyperinnervation peaks at 7 days postinfarction (15, 46, 51).

Hemodynamic Measurements

An echocardiographic expert performed the measurement in a blinded manner. For hemodynamics measurements, a polyethylene catheter was inserted into the left external jugular vein for fluid administration. A 2-Fr microtip P-V catheter (SPR-869; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the ascending aorta to measure left ventricular (LV) systolic and diastolic pressure as the mean of measurements of five consecutive pressure cycles with the use of a special P-V analysis program (PVAN, Millar Instruments) (18, 36).

In Vivo Electrophysiological Experiments

At the beginning of the test, the rats were endotracheally intubated and mechanically ventilated as described above. After thoracotomy, a programmed stimulation protocol was performed using electrodes implanted on the epicardial surface of the LV (50). Induced arrhythmias were effected using an electric Bloom stimulator (Chengdu Electronic Machine). The induction of VAs was then attempted by ventricular stimulation at a basic cycle length of 150 ms (S0) with single (S1), double (S2), and triple (S3) extrastimuli delivered after 8 paced beats. Pacing protocols were interrupted if sustained ventricular tachycardia was induced. Ventricular tachyarrhythmias, including ventricular tachycardia and ventricular fibrillation, were considered nonsustained when they lasted <15 beats and sustained when they lasted >15 beats. VA scores were determined by the inducibility quotient of ventricular tachyarrhythmias as follows: 0, noninducible; 1, nonsustained tachyarrhythmias induced with three extrastimuli; 2, sustained tachyarrhythmias induced with three extrastimuli; 3, nonsustained tachyarrhythmias induced with two extrastimuli; 4, sustained tachyarrhythmias induced with two extrastimuli; 5, nonsustained tachyarrhythmias induced with one extrastimulus; 6, sustained tachyarrhythmias induced with one extrastimulus; 7, tachyarrhythmias induced during a train of eight stimuli (8 × S1) at a basic cycle length of 120 ms; and 8, heart stopped before programmed electrical stimulation. When multiple forms of tachyarrhythmias occurred in one heart, the highest score was used (24). The experimental protocols were typically completed within 10 min. All procedures were executed and recorded using an animal biological function experiment system (LEAD-7000; JIET, Chengdu, China).

Histological Preparation

At the end of each experiment, the animals were killed by intravenous KCl injection. Cardiac tissue was then divided into four distinct areas: 1) right ventricle wall, 2) LV posterior wall (remote zone), 3) border region to the left anterior descending area [border zone, 3-mm zone adjacent to the MI border (38)], and 4) central zone of the infarcted area. The tissues were snap-frozen and stored separately at ~80°C for further biochemical analysis or were fixed in 10% zinc formalin or embedded in optimal cutting temperature compound (OCT) for histological examination.

Isolation of Monocyte from the Infarcted Area

Three days after surgery, infarcted tissue was pooled, minced, and digested three times in cell extraction buffer [1 mg collagenase IV, 1 mg dispase, and 0.5 mg hyaluronidase/1 ml of ADS buffer (0.11 M NaCl, 5.00 mM KCl, 5.00 mM dextrose, 0.80 mM MgSO₄, 0.02 mM CaCl₂, and 0.10 mM NaN₃)].
12.50 mM NaH₂PO₄, 20.00 mM HEPES)]. After each digestion, the supernatant containing the single-cell solution was removed, and fresh enzyme solution was added. During cell extraction, the solution was immersed in a shaking water bath at 37°C and bubbled with carbogen gas throughout the entire process. The single-cell solution was cooled to and subsequently handled at 4°C (42). Erythrocytes were lysed using red blood cell lysis buffer (50 mM NH₄Cl, 10 mM KHCO₃, 100 μM EDTA). The remaining cells were then incubated with anti-CD11b microbeads and anti-CD11b-APC (Biolegend, San Diego, CA).

**BM Isolation and Culture**

BM was isolated from the femurs and tibias of rats and cultured overnight in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. Nonadherent cells were collected by centrifugation and cultured at 1.0 × 10⁶ cells/ml in the presence of 20 ng/ml macrophage-colony stimulating factor (rat recombinant M-CSF; 50 ng/ml; Sigma-Aldrich) to generate BM-derived, M-CSF-dependent macrophages (BMM). After 3 days of culture, fresh media was added. Cultured macrophages were stimulated with LPS (10 μg/ml) and IFN-γ (20 ng/ml) or with 10 ng/ml recombinant rat IL-4 (Peprotek, Rocky Hill, NJ) for 24 h in the presence or absence of DAPT (10 μg/ml) (31, 34, 47). Supernatants were stored for cytokine analysis by ELISA, and the cells were harvested for RNA analysis.

**Quantitative Real-Time PCR**

Total RNA was isolated from LV muscles, macrophages were isolated from infarcted myocardium, and BMM cells were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Candidate gene expression was measured by quantitative real-time PCR using a PrimeScript RT reagent kit (Pierce, Madison, WI). Equal amounts of total protein (80 μg) were stored for cytokine analysis by ELISA, and the cells were isolated from infarcted myocardium, and BMM cells were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Each measurement was performed with gene-specific primers and SYBR Green 1 (Invitrogen, Carlsbad, CA). For each sample, both GAPDH and the 18S expression was measured by quantitative real-time PCR using a LightCycler 480 system (Roche). Each measurement was performed with gene-specific primers and SYBR Green 1 (Invitrogen, Carlsbad, CA). For each sample, both GAPDH and the 18S expression was measured by quantitative real-time PCR using a LightCycler 480 system (Roche). Each sample was measured in triplicate, and the data were analyzed using the 2⁻ΔΔCt method (46) for comparison between groups. The primers for each gene used in this study were as follows: Dll4: forward, 5'-GGCTGAGGTGGACATCTGC-3', reverse, 5'-CTGGCACACTTCTGAGTTC-3'; Notch1: forward, 5'-CCCGGTGTGAGAATGGT-3', reverse, 5'-CCCTGTAACCTTCTGTGC-3'; Hes1: forward, 5'-AAGTGGAGTGAGGTGTTCT-3', reverse, 5'-GGTTGATGGACTGGAC-3'; Arg1: forward, 5'-TTCCTCAAGATTACCTACCTC-3', reverse, 5'-GCTTTCT-3'; Cd163: forward, 5'-AAGTTGTTGGATCTGC-3'; reverse, 5'-CTGATCTCTACCCACTACATGG-3'; iNOS: forward 5'-GGTCCAACCTGCAGGTCTTC-3', reverse: 5'-CTGATCTCTACCCACTACATGG-3'; MMP-9: forward, 5'-GGCTGAGGTGGACATCTGC-3', reverse, 5'-CCCGGTGTGAGAATGGT-3'; GAPDH: forward, 5'-ACCTACCAAGCGGAGTTGAC-3', reverse, 5'-TCCCTCAAGATTACCTACCTC-3'; iNOS: forward, 5'-GGTCCAACCTGCAGGTCTTC-3', reverse: 5'-GGTTGATGGACTGGAC-3';

**Western Blotting**

For immunoblot analyses, proteins were isolated using a Protein Extraction Kit (Beyotime Institute of Biotechnology, Jiangsu, China). Extracted protein was measured with the BCA protein assay reagent kit (Pierce, Madison, WI). Equal amounts of total protein (80 μg of protein/lane) were resolved on 5–10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 and incubated overnight at 4°C with primary antibodies against NICD1 (1:500; Cell Signaling, Beverly, MA), Hes1 (1:800; Santa Cruz Biotechnology, Santa Cruz, CA), Arg1 (1:1,000; Abcam, Cambridge, MA), iNOS (1:500; Abcam), or NGF (1:1,000; Santa Cruz Biotechnology). The blots were developed using an enhanced chemiluminescence detection kit (Millipore, Billerica, MA) and visualized using a FluorChem E Imager (Protein-Simple, Santa Clara, CA). The densities relative to GAPDH were analyzed using NIH ImageJ software.

**Immunohistochemistry**

Hearts from each group were harvested and processed as standard formalin-fixed, paraffin-embedded tissues for histological study (21). Infarct size was evaluated on heart tissue sections stained with Masson’s trichrome according to standard protocols (7). The digitized pictures were analyzed by planimetry, and the infarcted area was expressed as the percentage of stained fibrosis area over total LV. For immunohistochemical staining of tissue, anti-NICD1 Ab (1:200; Cell Signaling), anti-α-smooth muscle actin (SMA) Ab (1:500; Abcam), or anti-Cd163 Ab (1:200; Abcam) antibodies were used along with appropriate secondary Abs (Vector Laboratories, Burlingame, CA). Following incubation with an ABC Elite kit (Vector Laboratories), slides were developed with a diaminobenzidine substrate kit (Vector Laboratories) and counterstained with hematoxylin. We used the number of CD31-positive vessel cells or α-SMA-positive vessel cells to assess the densities of capillaries and arterioles, the number of CD31-positive vessel cells or α-SMA-positive vessel cells to assess capillaries or arteriole density, and the number of α-SMA-positive cells to identify sum myofibroblast, taking care to exclude α-SMA-positive vascular smooth muscle.

For immunofluorescence, hearts were harvested after the hemodynamics study and immersed in 30% sucrose in PBS overnight, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and frozen in an isopentane bath on dry ice. Samples from the infarcted border zone were incubated with anti-tyrosine hydroxylase Ab (1:400; Millipore), anti-growth-associated protein 43 (GAP-43) Ab (1:200; Abcam), anti-ED1 (CD68) Ab (1:150, Abcam), anti-ED2 (CD163) Ab (1:100, Santa Cruz Biotechnology), anti-Arg1 Ab (1:100, Abcam), anti-NF Ab (1:50; Abcam) overnight at 4°C followed by a 2-h incubation with FITC-conjugated rabbit anti-sheep (1:200; Bethyl Laboratories, Montgomery, TX), Alexa 545-conjugated goat anti-rabbit (1:100; Peprotek), or FITC-conjugated rabbit anti-mouse (1:200; Biolegend) secondary antibodies. The sections were counterstained with DAPI (Life Technologies, Grand Island, NY) to identify nuclei. For quantification of the fraction of sympathetic nerve fibers, we analyzed four sections adjacent to those stained with Masson’s trichrome; the first section used for analyses was taken 1.5–2.0 mm apical to the ligation (44). The density is expressed as the ratio of labeled nerve fiber area to total area, whereas papillary muscles were excluded from the study because a variable sympathetic innervation has been reported (23). We used the number of CD68- and CD163-positive cells per CD68-positive cells as well as the number of CD68- and Arg1-double-positive cells per CD68-positive cells to correlate the M2/M polarization (12, 28). The contribution of macrophages to NGF expression within the infarct was assessed by the proportion of CD68-positive cells with NGF/CD68 colocalization (i.e., yellow in merged images) divided by the total number of CD68-positive macrophages.

A total of 10 microscopic fields in a ×20 field (0.13 mm²) were randomly selected by a blinded investigator. All images were obtained using an Olympus LCX100 Imaging System. All images were analyzed with ImageJ software (version 1.38x; National Institutes of Health, Bethesda, MD).

**ELISA**

A double-antibody sandwich ELISA kit (USCN Life Science, Wuhan, China) was used to detect serum TNF-α and IL-1β concentrations according to the manufacturer’s instructions. The intra- and intersample variability for each kit was <8%. Although cardiac innervation was detected by immunofluorescence staining of tyrosine hydroxylase and growth-associated factor 43, this did not imply that
the nerves were functional. Thus, to examine the sympathetic nerve function after administering DAPT, we measured LV norepinephrine levels from the infarcted border. The myocardia were minced and suspended in 0.4 N perchloric acid with 5 mmol/l reduced glutathione (pH 7.4), homogenized with a Polytron homogenizer for 60 s in 10 U of volume. Total norepinephrine was measured using a commercial ELISA kit (Noradrenalin ELISA; Immuno-Biological Laboratories, Hamburg, Germany).

Statistics

Data are presented as the means ± SD. Unpaired t-tests were used to compare values between two groups. Differences among more than two groups were compared using ANOVA followed by Tukey’s test. Analyses were performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). P values <0.05 were considered statistically significant.

RESULTS

Notch Signaling Pathway Is Activated in AMI

The immunohistochemical analysis of heart sections showed that a strong immunoreactive signal for NICD is detected at the infarct and peri-infarct area, primarily by inflammatory cells in the MI heart. On the other hand, the immunoreactive signal at the remote myocardium was almost undetectable (Fig. 1A). To assess the role of Notch signaling in MI, we then examined the expression of Notch1 mRNA in the heart tissue and isolated macrophages after MI. Monocytes/macrophages were successfully separated from the isolated cell population using magnetic cell sorting. More than 90% of the isolated cells showed a macrophage-specific antigen CD68 (Fig. 1B) in agreement with previously published results (10). We next investigated whether the upregulated Notch expression was derived by macrophage. Target genes were considered to be expressed by other cell types but not by macrophages if they showed decreased mRNA abundance in macrophages isolated from infarct zone compared with LV of sham-operated rats. Targets were defined as not predominated by macrophages if they did not show a strong increase (10-fold) in macrophages compared with the levels in the infarcted zone. In the entire infarcted zone, Notch1 increased 3.9-fold compared with the levels in the LV controls (Fig. 1C). Notch1 in macrophages increased 11.2-fold compared with the levels in the MI. Similarly, Hes1 increased 1.5-fold in the entire infarcted area compared with the levels in the LV controls and furthermore increased 10.5-fold in macrophages compared with the levels in the infarcted myocardium (Fig. 1C). This result indicates that the transcription of these genes is infarct-specific and derives predominantly from macrophages post-MI. Second, the immunofluorescence analysis revealed that NICD costained with macrophages and not myofibroblasts in nuclear and cytoplasma (Fig. 1, D and E). Moreover, the myocardium showed little NICD expression. Therefore, we speculate that Notch affected macrophage-dominated inflammation postinfarction.

Fig. 1. A: immunohistochemical staining of the Notch1 intracellular domain (NICD1) in sham-operated tissue, myocardial infarction (MI)-infarcted zone, MI-infarcted border, and remote zone (n = 5). B: after the isolation of macrophages, the cell population was spotted onto slides and immunostained using CD68 antibodies (green). Note that the majority (90%) of isolated cells are macrophages (nuclei: DAPI, blue). C: qRT-PCR of Notch1 and Hes1. Fold changes are expressed relative to mRNA abundance in the sham left ventricular tissue, which was set to 1. Both of the targets, activated Notch1 (NICD1) and Hes1, are strongly expressed in macrophages (MAC) compared with MI, BZ (border zone), RZ (remote zone), and left ventricle (LV) (n = 8). D: immunofluorescence costaining for NICD1 with CD68 (left) and myofibroblast (right) of infarcted tissue 3 days after MI (n = 5). Bar = 30 μm. Each column with a bar represents the mean ± SD. *P < 0.05, **P < 0.01.
Notch Blockade for 7 Days Exacerbates Cardiac Dysfunction and Ameliorates Sympathetic Innervation

We next assessed protein levels of NICD or the downstream target protein Hes1 by Western blot to evaluate the efficacy of DAPT. We observed that infarction was associated with a significant (3-fold) increase in NICD content (Fig. 2, B and D) and its downstream target Hes1 (Fig. 2, C and E) at 3 days post-MI. At 7 days post-MI, the expression level of NICD decreased drastically. The administration of DAPT prevented the activation of Notch signaling, as verified by the near-normal expression of NICD and the significant downregulation of Hes1. Following the successful establishment of the intervention, we tested this therapeutic approach in myocardial infarction. We observed that the infarct areas were larger in the MI-DAPT group than in the MI-DMSO group (58.4 ± 6.0% vs. 47.3 ± 5.0%, P < 0.05) (Fig. 2A), and they were accompanied by deteriorating cardiac function (Table 1). Densitometric quantification of α-SMA and CD31 protein indicated significantly less vessel formation in the MI-DAPT group (10.50 ± 1.50% vs. 6.30 ± 0.80%, 6.00 ± 0.50% vs. 2.50 ± 0.45%, P < 0.01).

Because of the importance of the inflammatory response in the regulation of post-MI remodeling, we explored the level of expression of proinflammatory cytokines. As expected, Notch inhibition alerted an inflammatory state post-MI, indicated by decreased expression profile of M1 cytokines (Fig. 1, F and G), confirming that Notch affected macrophage-dominated inflammation postinfarction.

Confirming the reduction in the level of NICD at the protein and functional levels, we investigated whether Notch is functionally important in neural remodeling. We assessed the sympathetic nerve anatomy and function by analyzing immunofluorescent staining and myocardial noradrenaline levels. The tyrosine hydroxylase-immunostained nerve fibers appeared to be oriented along the longitudinal axis of adjacent myofibers. Our findings demonstrating the localization of GAP-43 and tyrosine hydroxylase are consistent with other animal models of myocardial ischemia (23, 24). Significant sprouting of sympathetic nerve fibers was observed in the MI-DMSO rats; in contrast, administration of DAPT significantly reduced nerve density at the infarcted border zone (0.190 ± 0.045% in DAPT vs. 0.190 ± 0.045% in DAPT vs.

Fig. 2. A: original color images of Masson’s trichrome staining of the infarcted area in sham-DMSO (i), sham- N-N-(3,5-difluorophenacyl-L-alanyl)-S-phenylglycine-t-butyl ester (DAPT) (ii), MI-DMSO (iii), and MI-DAPT (iv). Representative expression levels of NICD1 (B and D) and Hes1 (C and E) at 3 days post-MI by Western blot (n = 5). Molecular weight markers are marked in the left of each band to show protein size (kDa). F and G: serum cytokine levels of IL-1β and TNF-α (n = 8). Each column with a bar represents the mean ± SD. **P < 0.01, *P < 0.05 compared with sham; †P < 0.05 compared with vehicle.
0.420 ± 0.140% in vehicle; P < 0.05) (Fig. 3). Similarly, the density of GAP-43 (Fig. 4), the marker for general neuronal sprouting, was also significantly attenuated in the DAPT-treated infarcted rats compared with the vehicle-treated infarcted rats (0.14 ± 0.03% in DAPT vs. 0.36 ± 0.13% in vehicle; P < 0.05). These morphometric results mirrored those of norepinephrine levels.

Effect of DAPT on Macrophages, Myofibroblasts and NGF Synthesis

To determine whether neural remodeling was associated with macrophage inflammation mediated by Notch signaling, we assessed the characteristics of the early macrophage subtype in the infarcted zone. Mass of infiltrated cells showed positive staining for the macrophage marker CD68 (Fig. 5, A–D) consistent with previous results. Measurements of CD68-positive cells that were costained for CD163 and Arg1 markers corroborate the numbers of M2 macrophages that were counted in each region at day 3 because this time period is when macrophages differentiate within the myocardium (39). As a result, decreased CD163-positive and Arg1-positive macrophage proportions and mRNA levels in the infarct area were observed (Fig. 5D). The number of a-SMA-positive myofibroblasts decreased slightly.

We then assessed the effect of Notch inhibition on NGF, a secretory protein that links macrophage and sympathetic innervation. Following MI, all of the CD68-positive macrophages expressed NGF, whereas the proportion of NGF-positive macrophages was decreased in the DAPT-treated rats of the high-dose group (10 mg/kg) and low-dose group (5 mg/kg) compared with the vehicle group in a dose-dependent manner (70.67 ± 5.73% vs. 48.86 ± 5.73%, P < 0.05) (Fig. 6, B and C). We then tested overall NGF expression. Both the mRNA and protein expression levels of NGF in both the infarcted border and the remote zone were significantly decreased, but high-dose DAPT was more efficient at suppressing NGF (Fig. 6, E and F, P < 0.05).

In Vitro LPS Stimulation Skewed Macrophages Pretreated with DAPT Toward M1 Polarization and Decreased NGF Production

Because we observed changes in the macrophage recruitment and functionality in rats, we wanted to characterize Notch expression and signaling in isolated macrophages in the context of stimulation. Additionally, we wanted to determine whether NGF induction is induced by Notch signaling. Confirmed by FACS analysis that most cultured BM-derived cells were macrophages, the LPS/IFN-γ-mediated BM macrophages shifting toward the M1 phenotype marked with the

Table 1. Cardiac morphology, hemodynamics, norepinephrine concentrations at the end of the study

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<th>Parameter</th>
<th>Sham Surgery</th>
<th>Ligation Surgery</th>
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Values are means ± SD. *P < 0.05 compared with respective sham; †P < 0.05 compared with vehicle-treated infarcted groups. DAPT, N-N-(3,5-difluorophenacyl-L-alanyl)-S-phenylglycine-t-butyl ester; EF, ejection fraction; LVESP, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; NE, norepinephrine.
upregulation of iNOS, whereas the IL-4-induced M2 phenotype differentiation marked with an increase in Arg1 expression (Fig. 7A). Moreover, evidence of Notch signal activation was observed in both the classically activated and the alternatively activated macrophages (Fig. 7, B–D).

We next sought to determine whether the blockade of Notch signaling using DAPT, which prevents the cleavage and nuclear translocation of Notch receptors, would inhibit the expression of NGF. The induction of NGF by LPS/IFN-γ stimulation decreased when macrophages were co-cultured with 10 μg/ml DAPT, and this treatment promotes the switch to alternatively activated infiltrating macrophages (Fig. 7F). Therefore, Notch signaling regulates NGF downstream of M1 macrophages in response to LPS/IFN-γ. In contrast, the levels of NGF secretion in response to IL-4 were slightly but not significantly decreased by DAPT although Notch signaling is induced by IL-4 treatment (Fig. 7F), indicating that the induction of NGF by IL-4 does not depend on Notch signal activation. Meanwhile, protein expression of arginase was induced in response to IL-4 but unaffected by Notch inhibition (Fig. 7E). These results suggest that the Notch-mediated protein expression of NGF is not associated with the M2 phenotype.

**Electrophysiological Stimulation**

To further elucidate the physiological effect of attenuated sympathetic hyperinnervation, ventricular pacing was performed. The arrhythmia score in sham rats was 0 (Fig. 8). Ventricular tachyarrhythmias were inducible by programmed stimulation in infarcted rats. DAPT treatment significantly decreased the inducibility of ventricular tachyarrhythmias in infarcted rats compared with vehicle treatment (both P < 0.05).

**DISCUSSION**

To our knowledge, the present study is the first to elucidate the Notch-dependent molecular and cellular inflammatory changes associated with MI. Notch1 in adult myocytes does not contribute to cardiac repair because of permanent epigenetic modifications at Notch-responsive promoters, whereas the critical role of Notch signaling in BM-derived cells following myocardial injury has been recently emphasized (9, 25). We explored the functional consequences of Notch inhibition on activity in macrophages, a cell type central to the postinfarction process, and further evaluated the effect of Notch inhibition on neural remodeling.

Inflammation is part of the physiological wound-healing response following mechanical lesioning of the peripheral nervous system, and the proinflammatory cytokines IL-1β, TNF-α, and IFN-γ can provide a neuroprotective signal via a cytokine/neurotrophin axis (14). In contrast, generalized down-regulation of cytokine expression leads to suboptimal axonal plasticity at distal nerve sites (20). The causative effect of inflammation on sympathetic reinnervation postinfarction has also been demonstrated in experimental studies, whereas either macrophage depletion or the inflammation inhibition by an NF-κB inhibitor, as well as other anti-inflammatory agents, could block this process and ameliorate nerve sprouting and sympathetic hyperinnervation (8, 44, 49). A key link between neural remodeling and inflammation postinfarction has been attributed to macrophages, which are the major immune cells to modulate neuronal survival and nerve fiber outgrowth. Macrophages are attracted to injured nerve fibers immediately following tissue damage and then secrete cytokines that can regulate neuronal gene expression and neurotrophic factors (4). The following cells are thus potential therapeutic targets in animal experiments: injection of monocytes/macrophages into the immediate surroundings of peripheral nerve cell bodies significantly enhances axonal regeneration (22); in contrast, macrophage depletion post-MI markedly reduces cardiac sympathetic hyperinnervation (46). Recently, Zhang et al. (50) reported that macrophages play a key but transient role in nerve regeneration at an early stage post-MI after the injection of Schwann cells. However, the underlying mechanism remained unknown.

Notch signaling is most widely studied in immune cells, specifically in the juxtacrine homotypic communication between macrophages as well as in the amplification of the proinflammatory milieu in inflamed tissues in various ischemia injury models (30, 48). Increased systemic and local macrophage chemotaxis leads to the activation of Notch signaling and predominantly classically activated macrophages (M1) and prolongs the recruitment of inflammatory cells to the wound (34). Phenotypic changes during the important transition into an alternatively activated type (M2) are equally critical in the resolution of inflammation by producing anti-inflammatory cytokines and chemokines and by increasing phagocytic activ-
The development of Dll4 antibodies or \( \gamma \)-secretase inhibitors may be a novel therapeutic approach to autoimmune disease and injury progression (29, 41). In our study, enhanced Notch signaling was documented at early stages, primarily in macrophages. The administration of DAPT, a \( \gamma \)-secretase inhibitor that inhibits all Notch1-4 signals, abolished the upregulation of NICD and Hes1 in the infarcted tissues and altered the inflammatory state by decreasing cytokine expression, reducing macrophage infiltration, and shifting the macrophage activation subtypes. Specifically, the administration of DAPT

Fig. 5. Tissues were harvested for immunofluorescence at 3 days postinjury from vehicle-treated or DAPT-treated rats. Representative double-immunostained images for CD68 (red) as a macrophage marker, costained for CD163 (green) or arginase (green) as an M2 macrophage, merged images, and DAPI (blue) for nuclei in the MI-DMSO group (A and C) and the MI-DAPT group (B and D) (n = 5). Proportions and relative mRNA levels of CD163 and arginase are expressed in infarcted tissues (E and F). Values shown are the mean ± SD. **P < 0.01 compared with sham; †P < 0.05 compared with vehicle.
Fig. 6. The dose-dependent effect of DAPT on nerve growth factor (NGF) protein expression. Double immunostaining for CD68 (green) as a macrophage marker and costained for NGF (red), merged images (n = 5) in vehicle-treated (A), low dose-DAPT-treated (B), or high-dose-DAPT-treated rats (C). Bar = 30 μm. D: percentage of NGF immunoreactivity macrophages. E and F: RT-PCR analysis and Western blot of NGF in homogenates of the LV from the infarcted border zone (n = 5), and each mRNA was corrected for the mRNA level in vehicle-treated rats. Molecular weight markers are marked in the left of each band to show protein size (kDa). Results are shown as the means ± SD of 3 independent experiments. *P < 0.05 compared with infarcted groups treated with vehicle; †P < 0.05 compared with the low-dose DAPT-treated infarcted group. L, low-dose DAPT (5 mg/kg per day); H, high-dose DAPT (10 mg/kg per day).
increased M2 macrophage infiltration at 3 days post-infarction, which is earlier than expected, and dysregulated the M1/M2 balance. Otherwise, the NICD and Hes1 levels decreased dramatically at day 7 post-MI in parallel with the transition from M1 to M2 macrophages, as previously observed in healing infarcts (6, 22). Therefore, we concluded that Notch inhibition reduces M1 polarization at early stages and promotes their M2 fate in vivo, causing a state of decreased inflammation. Moreover, this inhibition did cause a modest reduction in the wound area occupied by a-SMA-positive myofibroblasts, which may occur secondarily to the macrophage inflammatory status, as the majority of the a-SMA-positive myofibroblasts developed from BM-derived cells (2, 26). These results led us to explore whether Notch-mediated macrophage shifting could regulate sympathetic hyperinnervation.

The density of GAP-43-positive and tyrosine hydroxylase-positive nerve fibers was clearly decreased, and hyperinnervation was prevented by Notch inhibition in the infarcted border zone. Sympathetic hyperinnervation fails to develop at the site of the ischemic injury. This is not likely to be due to an effect of DAPT on sympathetic innervation because sympathetic nerve density was unaffected in sham-ligation subjects. We then investigated whether DAPT affects neural remodeling via NGF, a neurotrophic factor critical for promoting the aggressive sprouting of sympathetic nerves (3, 27). Consequently, DAPT significantly decreased the proportion of NGF-positive macrophages and markedly reduced the high expression of NGF in a dose-dependent manner, which was accompanied by reduced levels of macrophage-produced cytokines, such as IFN-γ and IL-β. This result can be explained by the fact that macrophages may be more involved in the inflammatory stage (M1) rather than the reparative stage (M2) under neuropathological conditions, including the release of inflammatory cytokines, which may indirectly reduce sympathetic outgrowth by modulating NGF but are not known to directly elicit sympathetic sprouting (40). The shifting of different phenotypes is pivotal for the removal of cellular debris and for ultimately assisting neuron fiber sprouting, but the underlying mechanism is poorly understood. We then assessed Notch activity in macrophages in response to classical or alternative activation. However, as reported previously, stimulation with both LPS and IFN-γ induced Notch activation in our assays. Exposure to LPS/IFN-γ upregulated the surface expression of NGF in a Notch-dependent manner. Notch inhibition acts on the M1 subtype to reduce NGF secretion, whereas the M2 subtype does not appear to be involved in that process. However, none of these strategies have been translated into the clinic (33).

It seems that Notch activation plays an important role in the dynamic and superbly orchestrated process of innate immunity-induced neural remodeling post-MI. The present data are supported by studies of Notch in neuroinflammation in central nervous system-induced neural remodeling post-MI. The present data are supported by studies of Notch in neuroinflammation in central nervous system.
nerve ischemia (43, 45). Interestingly, we also found that DAPT potentially decreased angiogenesis at both capillary and arteriolar levels and contributed to augmentation of the infarct size and aggravation of cardiac dysfunction postinfarction despite its crucial role in alleviating the inflammatory reaction, supported by a previous study demonstrating increased collagen deposition and decreased vessel formation in Notch1-positive/negative wounds, although M2 expression was not addressed (34). These data indicated that cardiac remodeling was more pronounced in the absence of Notch signal. The M1/M2 balance dysregulation, with an excess of M2 macrophages at an earlier than expected time frame, was most likely to contribute the impaired repair mechanisms following MI. Our data are supported by the evidence that M2 macrophages have been characterized as being profibrotic, and early shifting of macrophage phenotype to M2 exacerbates cardiac remodeling (39). The findings above hint that macrophage phenotypic balance between M1 and M2 facilitates a complete cardiac repair response post-MI. It could therefore be beneficial to modulate the timing of the recruitment or the ratio of macrophage subtypes to emphasize tissue repair. In addition, the malignant outcomes may rely on the suppression of the Notch pathway on multiple types of targeted cells, including endothelial cells and inflammatory cells. As previous studies confirmed the beneficial effect of Notch1 in BM-derived inflammatory cells in cardiac repair, particularly through the secretion of growth factors that could promote neovascularization and prevent cardiomyocyte apoptosis in the infarct border zone (25), we are focusing on myeloid-specific Notch1-deletion techniques and targeted cell transfusion of BM transplantation to demonstrate whether the macrophage-dominated inflammatory response is the main regulatory method and whether selectively controlling the expression of Notch after MI may have pleiotropic benefits.

Taken together, our models have established a specific role for Notch in macrophage recruitment and function postinfarction. Future studies should use selective myeloid inhibitors and inflammatory mediators to further elucidate each step of the pathway from macrophage polarization to sympathetic sprouting. A clarification of the mechanism of nerve regeneration, which may be improved greatly by the Notch inhibition, will require the exploration of correlation factors in the Notch signaling system at an extensive range of time points following the operation and a more precise understanding of the appropriate dosage and timing for the administration of these drugs.

Study Limitations

1) The presented data are collected from a permanent ligation model without reperfusion, which is different from the clinical setting. Furthermore, more investigations are required in larger animal models and suitably aged animal hearts. 2) Macrophages from humans and rats may have different programs because variations in character have influence in many disease settings. 3) Although γ-secretase inhibitors may be useful in screening for involvement of the Notch signaling pathway, genetic approaches such as knockdown or overexpression studies are necessary for more definitive conclusions. 4) As γ-secretase inhibitor target patients gene expression in multiple cell types involved in cardiac injury, further study is required to clarify how Notch activity in other cell types may contribute to cardiac remodeling. Despite such limitations, however, our study clearly shows that the expression level of Notch signaling in infiltrated macrophages is higher than that in cardiomyocytes and fibroblasts and points toward a key pathologic role of the Notch signaling pathway in postinfarction remodeling.

Conclusion

This study demonstrates a novel role for Notch signaling in sympathetic hyperinnervation post-MI. The mechanism of action of Notch was mediated partly via macrophage-mediated regulation of NGF and cytokine expression.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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