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Preventive Effect of Notch Signaling Inhibition by a γ-Secretase Inhibitor on Peritoneal Dialysis Fluid-Induced Peritoneal Fibrosis in Rats

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Peritoneal fibrosis, a major complication of peritoneal dialysis, limits the effectiveness of peritoneal dialysis as a treatment of end-stage renal disease. Preventing this complication by identifying targets for therapy has recently received much attention. In the present study, we showed that Notch signaling was highly activated in rats in peritoneal dialysis fluid-induced fibrotic peritoneum, as indicated by increased expression of Jagged-1, Notch-1, and HES-1. Blocking Notch signaling activation by intraperitoneal injection of a γ-secretase inhibitor, DAPT, significantly attenuated peritoneal fibrosis as indicated by the decreased expression of α-smooth muscle actin, collagen I, and vascular endothelial growth factor as well as increased expression of E-cadherin. Moreover, compared with control rats, DAPT-treated rats had a thinner peritoneum with less extracellular matrix accumulation, a lower mass transfer of glucose, and a higher ultrafiltration rate. In addition, transforming growth factor (TGF)-β1 induced Notch signaling activation in primary rat peritoneal mesothelial cells. DAPT blocked this TGF-β1–induced Notch signaling activation and therefore significantly inhibited TGF-β1–induced expression of α-smooth muscle actin, collagen I, and vascular endothelial growth factor. Thus, a γ-secretase inhibitor that interferes with Notch signaling prevents biochemical, histological, and functional consequences of peritoneal fibrosis through inhibiting epithelial to mesenchymal transition of rat peritoneal mesothelial cells. These results support the use of γ-secretase inhibitors as a novel therapeutic approach for peritoneal fibrosis. ***(Am J Pathol 2010, 176:650–659; DOI: 10.2353/ajpath.2010.090447)***

Peritoneal dialysis (PD) is a convenient and inexpensive therapy for patients with end-stage renal disease. In long-term PD, the effectiveness is markedly limited mainly by the fibrotic changes in the peritoneal membrane.[1,2] Thus, there is a pressing need for the understanding of the molecular pathogenesis of peritoneal fibrosis and the development of effective therapy for preventing peritoneal fibrosis.

The monolayer of peritoneal mesothelial cells is the key structure of the biological and physical barrier that are involved in regulating permeability and ultrafiltration in PD.[3] In patients chronically exposed to the peritoneal dialysis fluid (PDF), there is a loss of mesothelial cells and the replacement of the peritoneal membrane by fibrous tissue.[4,5] Recent studies revealed an important role of mesothelial cells in peritoneal injury through the epithelial-to-mesenchymal transition (EMT) induced by PDF. Submesothelial myofibroblasts, which participate in extracellular matrix accumulation (ECM) and angiogenesis, can originate from mesothelial cells through EMT.[6,7] Therefore, EMT is an early event in peritoneal membrane fibrogenesis and is likely mediated by transforming growth factor (TGF)-β both in mesothelial cell culture and *in vivo*.[8,9]

Notch signaling is an ancient cell signaling system that regulates cell fate specification, stem cell maintenance, and initiation of differentiation in embryonic and postnatal tissues.[10–12] Four Notch receptors isoforms, namely Notch-1,[13] Notch-2,[14] Notch-3,[15] and Notch-4,[16] and five ligands, Jagged-1[17] and Jagged-2[18] belonging to the Serrate family, and Delta-1,[19] Delta-3,[20] and Delta-like 4[21] belonging to the Delta family, have been identified in mammals. The pathway is activated through an interaction of a Notch receptor with a Jagged or Delta-like ligand leading to proteolytic cleavages of Notch receptor at two

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distinct sites. The cleavage releases the Notch intracellular domain (NICD) such that it can enter the nucleus and function as a transcription activator. Importantly, the second cleavage is mediated by the γ-secretase complex, and effective inhibition of Notch activation can be achieved by pharmacological inhibition of this proteolytic activity. Once within the nucleus, NICD interacts with CSL (RBP-Jk/CBF1) and Mastermind to generate a large transcriptional activator complex and activates transcription of downstream target genes.[22] In mammals, primary target genes of the Notch-intracellular domain/RBP-J complex include the *HES* (Hairy/Enhancer of Split)[23,24] and *HEY* (HES-related with YRPW motif, also named HERP, HES-related repressor protein)[25–27] family of genes, which act as transcription factors.

Notch has recently been shown to promote EMT during cardiac valve formation.[28] Moreover, an upregulation of Notch ligand Jagged-1 expression was detected in the kidney of a model of progressive interstitial fibrosis induced by ureteral obstruction.[29] In epithelial cells from mammary gland, kidney tubules, and epidermis, TGF-β induces the Notch target gene *Hey1* at the onset of EMT in a Smad3-dependent process.[30] However, despite a most recent report showing expression of Jagged-1 in peritoneal mesothelial cells,[31] little is known about the expression pattern and functional role of the Notch signaling pathway in normal and injured peritoneum induced by long term PD.

In the present study, we investigated the role of Notch signaling in the progression of peritoneal fibrosis induced by PDF. Our results demonstrated that the components of Notch signaling are expressed and activated in fibrotic peritoneum induced by PDF. Moreover, TGF-β induced the expression of Notch signaling components during the process of EMT of primary rat mesothelial cells (RPMCs). Because γ-secretase inhibitor (GSI) has been extensively used for inhibiting Notch signaling both *in vitro*[32] and *in vivo*[33] and also been clinically tested for T cell acute lymphoblastic leukemia,[34,35] we used GSI to treat RPMCs and found that GSI dramatically inhibited TGF-β–induced EMT of RPMCs. Most importantly, we demonstrated that GSI could significantly attenuate peritoneum fibrosis and prevent a loss of peritoneal function *in vivo*. These results support the use of GSI as a novel therapeutic approach for PD-related peritoneal fibrosis.

Materials and Methods

Reagents and Antibodies

Antibodies against α–smooth muscle actin (α-SMA) and GAPDH were from Sigma-Aldrich (St. Louis, MO). Notch-1(sc-6015) and HES-1 antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibody specific for NICD (ab52301) was from Abcam (Cambridge, MA). Vascular endothelial growth factor (VEGF) antibody was purchased from Thermo Fisher Scientific Inc (Fremont, CA). Goat anti-type collagen antibody was obtained from Southern Biotech (Birmingham, AL). Jagged-1 antibody and secondary HRP-conjugated antibodies were from Cell Signaling (Danvers, MA). FITC-conjugated donkey anti-goat IgG antibody was purchased from Jackson Immuno Research Laboratories Inc (West Grove, PA). Fluorescent phallotoxin-labeled secondary antibodies were from Invitrogen (Carlsbad, CA). γ-secretase inhibitor DAPT was purchased from Calbiochem (San Diego, CA). Recombinant human TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Animal Experiments

Male Sprague-Dawley (SD) rats weighing 150 to 180 g were obtained from the Experiment Animal Centre at the Northern Campus of Sun Yat-sen University. All animal experiments were approved by the Committee on Animal Experimentation of Sun Yat-sen University and performed in compliance with the university's Guidelines for the Care and Use of Laboratory Animals. Thirty male SD rats were randomly allocated into five groups: rats in group A ($n = 6$) served as normal controls; rats in group B ($n = 6$) and group C ($n = 6$) received daily intraperitoneal injections of PDF named Dianeal® PD-2 Peritoneal Dialysis Solution with 4.25% Dextrose (4.25% Dianeal; Baxter HealthCare, Deerfield, IL) at 100 ml/kg of body weight[36]; rats in group D ($n = 6$) were intraperitoneally injected with 10 μmol/L DAPT together with 4.25% Dianeal; rats in group E ($n = 6$) received the same amount of DMSO (the vehicle for DAPT) as group D together with 4.25% Dianeal. Rats of group B were sacrificed at 14 days and the rest of rats were sacrificed at 28 days after initial treatment.

Peritoneal Function Test

Peritoneal function tests were performed as previously described.[37] Briefly, for the peritoneal ultrafiltration rate, 4.25% Dianeal was administered intraperitoneally to the rats at 90 ml/kg body weight before being euthanized. Four hours later, the peritoneal fluid was removed for ultrafiltration measurement. Net ultrafiltration was the volume of fluid removed after four hours minus the volume of fluid administered. For glucose transportation assay, glucose was measured by a standard enzymatic test on a Hitachi automated chemistry analyzer (Hitachi 7170, Japan). Mass transfer of glucose from the peritoneum was calculated using the formula: (initial dialysate glucose × initial volume) − (final dialysate glucose × final volume). These values were corrected for animal weight at the time of euthanasia.

Histopathological and Immunofluoresecence Analysis of Rat Peritoneum

Four-μm paraffin sections from the anterior abdominal wall were stained with hematoxylin and eosin and Masson trichrome. The thickness (μm) of the peritoneum was measured in each animal using a micrometer fitted into

the eyepiece of the microscope and expressed as the means ± SD. Each section was measured at 10 random sites.

For immunofluoresence analysis, 10-μm paraffin-embedded sections from the visceral peritoneum were cut and dehydrated in xylene, followed by microwave antigen retrieval. Sections were then blocked with PBS containing 5% BSA for 30 minutes at 37°C. Sections were incubated overnight at 4°C with indicated primary antibodies diluted in 3% BSA in PBS and secondary antibodies for 50 minutes at 37°C. Samples were mounted in fixation medium (Biomeda, Foster City, CA). DAPI (Sigma-Aldrich, St. Louis, MO) was used to stain the nucleus. Images were analyzed and collected with Zeiss LSM 510 Confocal Imaging System (Zeiss, Germany).

RPMCs Isolation and Culture

The isolation and culture of RPMCs was performed according to our previously reported method.[37] Briefly, RPMCs were prepared by infusing 30 ml of 0.25% trypsinase-0.2% EDTA-Na_2 into the rat abdominal cavity. The fluid was removed from the peritoneal cavity one hour later under sterile conditions. To harvest RPMCs, cellular components were isolated by centrifugation and then washed with PBS and suspended in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 12% (v/v) FCS (Invitrogen, Carlsbad, CA). Cells were placed into 25-cm^2 culture flasks and incubated overnight at 37°C. Nonadherent cells were removed the next day, and the adherent population was incubated at 37°C in 5% CO_2 in fresh culture medium. RPMCs in this study were derived from two to four passages grown as a monolayer to subconfluency.

To induce EMT, RPMCs were seeded into 35-mm diameter tissue culture plates. When 60% confluent, cells were cultured in serum-free medium for 24 hours and then indicated amount of TGF-β1 was added for various time period. To examine the effect of γ-secretase inhibitor DAPT on TGF-β1–induced EMT, RPMCs were pre-incubated for 15 minutes with 10 μmol/L DAPT before TGF-β1 treatment.

RT-PCR

Total peritoneal RNA was isolated from visceral peritoneum using Trizol Reagent (Invitrogen, Carlsbad, CA) and was reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) as previously described.[37] Amplified cDNA was used as the template DNA, and PCR was performed with TaqDNA polymerase and specific primers. The steps were as follows: denaturation at 95°C for 5 minutes followed by denaturation at 95°C for 30 seconds, hybridization at 60°C for 30 seconds, and elongation at 72°C for 45 seconds, for 25–32 cycles. The following PCR primers were used: Jagged-1: forward, 5′-GCCAAGTGGGATGACGACT-3′, reverse, 5′-GCAACAGCAGCGATAAGTGA-3′; Notch-1: forward, 5′-GCAAGAAGAAGCGGAGAG-3′, reverse, 5′-AGCTGGCACCCTGATAGATG-3′; HES-1: forward, 5′-CAGATGACCGCCGCTCTCA-3′, reverse, 5′-GCGACACTGCGTTAGGACCC-3′; GAPDH: forward, 5′-AGATCCACAACGGATACATT-3′, reverse, 5′-TCCCTCAAGATTGTCAGCAA-3′.

Western Blot Analysis

Tissue and cells were lysed in lysis buffer (50 mmol/L Hepes pH 7.5, 150 mmol/L NaCl, 10% glycerol, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_5, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 20 μg/ml aprotinin). Protein was quantified by the Bradford assay (Bio-Rad, Hercules, CA), equal amount of protein were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). After blocking in 5% skim milk for 1 hour at room temperature, membranes were incubated with indicated primary antibody at 4°C overnight followed by horseradish peroxidase-conjugated second antibody for 1 hour at room temperature and detected by chemiluminescence (Amersham Biosciences, Piscataway, NJ). Quantification of the Western blot data were performed by measuring the intensity of the hybridization signals using NIH Image analysis program.

Statistics

The results were expressed as mean ± SD. Statistical analysis was performed using SPSS13.0. Data were analyzed using one-way analysis of variance followed by post hoc test. A value of $P < 0.05$ was considered as statistically significant.

Results

The Notch Signaling Cascade Was Activated in a Rat Model of Peritoneal Fibrosis Induced by PDF

To investigate the expression of Notch signaling components in fibrotic peritoneum, we generated a rat model of peritoneal fibrosis induced by daily intraperitoneal injection of PDF containing 4.25% glucose at 100 ml/kg of body weight, and rats were sacrificed either 14 days or 28 days after the initial treatment. As shown in Figure 1, A and B, the mRNA levels of Jagged-1, Notch-1, and the Notch downstream target HES-1 was hardly detected in normal peritoneum by RT-PCR, but their expression was gradually increased in rats after 14 days of PDF treatment. The mRNA expression of Jagged-1, Notch-1, and HES-1 in 28 days group was readily detected and was significantly higher than that in control group. Similarly, the protein level of Jagged-1 and HES-1 was increased gradually after PDF treatment, and a significant increase was detected in 28 days group compared with that of control group (Figure 2, A and B). Because the cleavage of Notch-1 is an indicator of Notch signaling activation,

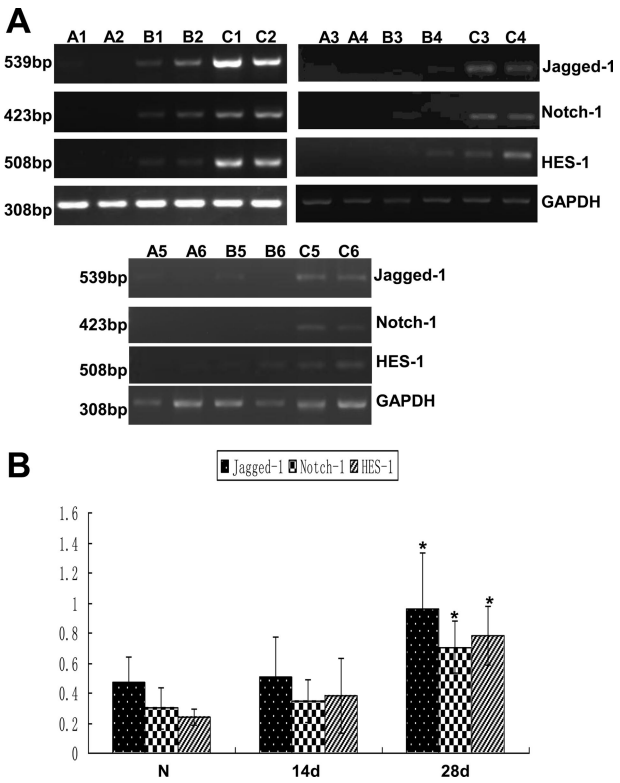

Figure 1. Transcripts of Notch signaling components were increased in fibrotic peritoneum induced by PDF. **A:** Normal rats (A1 to A6) or rats that received daily intraperitoneal injections of 4.25% Dianeal (100 ml/kg) for 14 days (B1 to B6) or 28 days (C1 to C6) were sacrificed, mRNA was collected, and RT-PCR was performed to detect mRNA level of Notch-1, Jagged-1, and HES-1. GAPDH was used to verify equivalent loading. Each panel represents an independent experiment with two animals per group. **B:** Graphic representation of relative abundance of Notch-1, Jagged-1, and HES-1 normalized to GAPDH. Data are given as mean ± SD ($n = 6$). $^{*}P < 0.05$ versus normal rats.

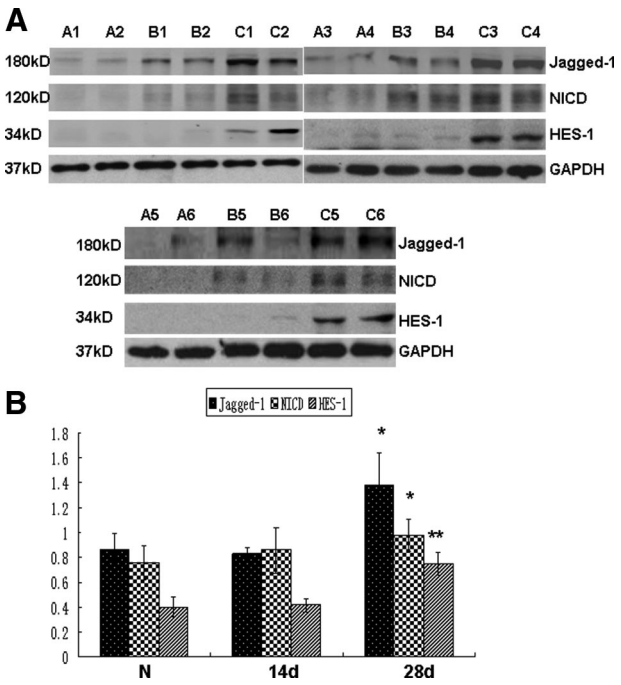

Figure 2. The protein level of Notch signaling components was increased in fibrotic peritoneum induced by PDF. **A:** Normal rats (A1 to A6) or rats that received daily intraperitoneal injections of 4.25% Dianeal (100 ml/kg) for 14 days (B1 to B6) or 28 days (C1 to C6) were sacrificed. The protein levels of NICD, Jagged-1, and HES-1 were analyzed by Western blot. GAPDH was used to verify equivalent loading. Each panel represents an independent experiment with two animals per group. **B:** Graphic representation of relative abundance of NICD, Jagged-1, and HES-1 normalized to GAPDH. Data are given as mean ± SD ($n = 6$). $^{*}P < 0.05$ versus normal rats. $^{**}P < 0.01$ versus normal rats.

we examined the protein level of NICD by Western blot and detected a significant increase of NICD in the peritoneum after 28 days of PDF treatment (Figure 2). The expression of NICD, Jagged-1, and HES-1 at the protein level was further documented by immunofluorescence. As shown in Figure 3, A–C, positive staining for NICD, Jagged-1, and HES-1 was readily detected in the mesothelial cells on the surface of the peritoneum and in cells in the submesothelial areas after 28 days of PDF treatment. In contrast, few cells with positive staining for NICD, Jagged-1, and HES-1 expression were detected in normal peritoneum. We next examined the localization of the upregulated HES-1 as a marker of activated Notch signaling by immunofluorescence. In the fibrotic peritoneum, the HES-1–positive staining was colocalized with α-SMA, a phenotypic marker of myofibroblast and a hallmark of EMT of mesothelial cells, in the cells on the surface of the peritoneum and in the submesothelial area (Figure 3D). These data indicated that the upregulation of Notch signaling occurs in myofibroblasts and transdifferentiated mesothelial cells. These data demonstrated that Notch signaling was highly activated in fibrotic peritoneum induced by PDF.

DAPT Attenuates PDF-Induced Peritoneal Fibrosis and Prevents a Loss of Peritoneal Function

Given the potential clinical implication of GSI, we further tested the possibility that blocking Notch signaling activity by GSI could attenuate peritoneal fibrosis *in vivo*. Rats were administrated with 10 μmol/L *N*-[*N*-(3, 5-difluorophenacetyl)-l-alanyl]-*S*- phenylglycine *t*-butyl ester (DAPT), a highly active GSI, by intraperitoneal injection together with 4.25% Dianeal (100 ml/kg) for 28 days. Control rats received the same amount of DMSO (the vehicle for DAPT) together with 4.25% Dianeal (100 ml/kg). The efficiency of DAPT on inhibiting Notch signaling was demonstrated by decreased protein level of NICD and HES-1 (Figure 4, A and B). As expected, peritoneal fibrosis developed on day 28 after PDF treatment that was characterized by a marked peritoneal thickening, significant ECM accumulation, and a loss of linear mesothelial cells in rats that received PDF alone and in control rats receiving both DMSO and PDF. However, these features of peritoneal fibrosis remarkably attenuated in DAPT-treated rats. As shown in Figure 5, A–C, the thickness of peritoneum was significantly reduced in the DAPT-treated rats accompanied by less ECM accumulation. Moreover, the rates of mass transfer of glucose (MTG) and ultrafiltration rate, representative indexes of peritoneal function, were preserved in the DAPT-treated rats in comparison with those treated with PDF (Figure 6, A and B). Moreover, the preservation of peritoneal function by DAPT was accom-

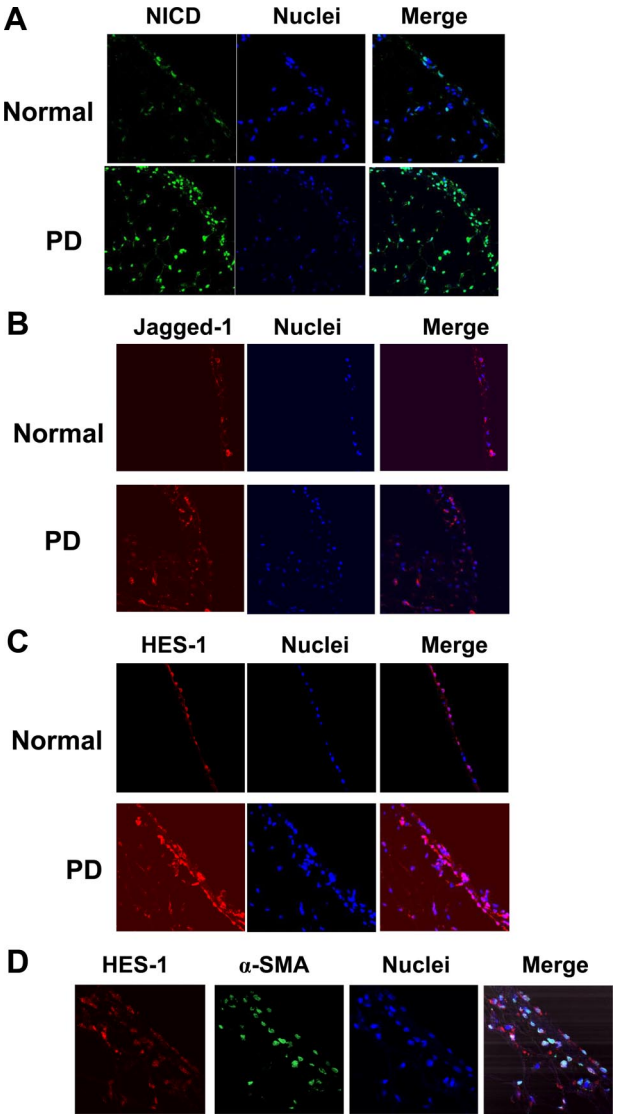

Figure 3. Immunofluorescence evidence for the increased Notch signaling activation in fibrotic peritoneum. Peritoneal sections of normal rats or rats on 28-day PDF treatment (100 ml/kg) were paraffin-fixed and stained with antibodies against NICD (**A**), Jagged-1 (**B**), and HES-1 (**C**). **D:** Peritoneal sections of rats on 28-day PDF treatment (100 ml/kg) were paraffin-fixed and costained with antibodies against HES-1(red) and α-SMA (green). Nuclei were stained with DAPI (blue). Images (magnification ×400) were taken by confocal microscopy.

panied by the attenuation in expression of several proteins characteristic of EMT and peritoneal fibrosis. By both Western blot analysis and immunofluorescence staining, the expression of α-SMA, collagen I, and VEGF was markedly attenuated in DAPT-treated rats compared with PDF treated rats, whereas the E-cadherin expression was increased significantly in DAPT-treated rats (Figure 7, A–C).

DAPT Attenuated TGF-β–Induced EMT of RPMCs

Prior studies demonstrated an important role of mesothelial cells in peritoneal fibrosis through EMT, and TGF-β could induce Notch downstream target Hey-1 expression in epithelial cells. Therefore, we hypothesized that TGF-β activates Notch signaling of RPMCs, and the mechanism by which DAPT prevents peritoneal fibrosis is by inhibiting EMT of RPMCs. To test this hypothesis, we isolated mesothelial cells from rat peritoneum and treated with incremental amount of TGF-β1 for 48 hours. As shown in Figure 8, A and B, the expression of NICD, Jagged-1, and HES-1 was gradually increased in a dose-dependent manner after TGF-β1

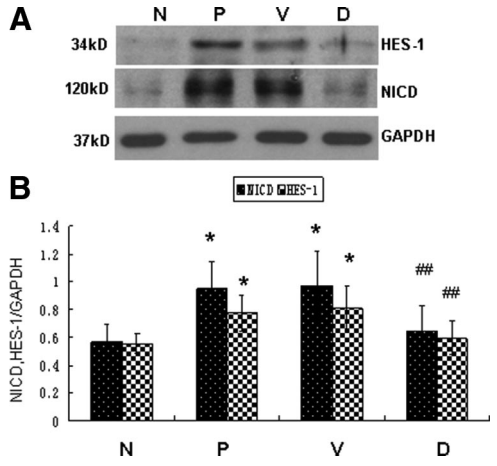

Figure 4. Effect of DAPT on inhibiting Notch signaling activation in fibrotic peritoneum. Peritoneal proteins were extracted, and the protein level of NICD and HES-1 was examined by Western blot. GAPDH was used to verify equivalent loading. **B:** Graphic representation of relative abundance of NICD and HES-1 normalized to GAPDH. Data are expressed as mean ± SD ($n = 6$). $^{*}P < 0.05$ versus normal rats. $^{\#\#}P < 0.01$ versus PDF-treated rats and vehicle control rats. N indicates normal rats; P, PDF-treated rats; V, vehicle control rats treated with DMSO together with PDF. D, Rats treated with DAPT together with PDF.

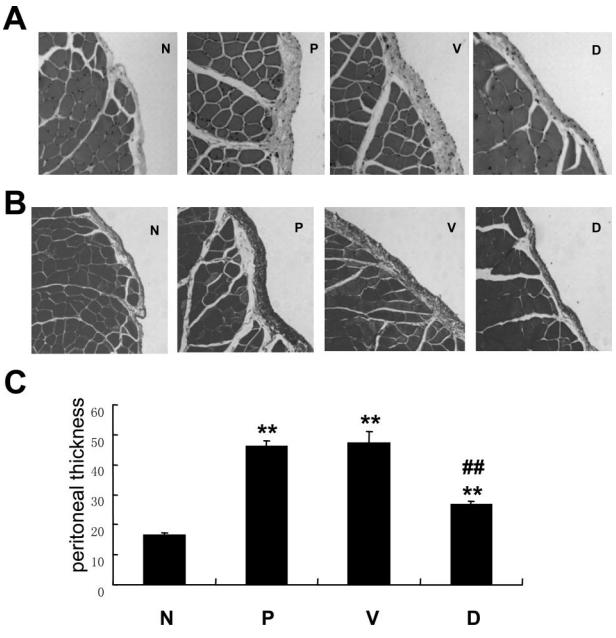

Figure 5. DAPT treatment attenuated peritoneal fibrosis induced by PDF. **A:** Hematoxylin and eosin staining of paraffin-fixed parietal peritoneal sections. **B:** Masson Trichrome staining of paraffin-fixed parietal peritoneal sections. **C:** Semiquantification of the thickness of peritoneal membrane. Data are mean ± SD ($n = 6$). $^{**}P < 0.01$ versus normal rats. $^{\#\#}P < 0.01$ versus PDF-treated and vehicle control rats. N indicates normal rats; P, PDF-treated rats; V, vehicle control rats treated with DMSO together with PDF. D, Rats treated with DAPT together with PDF. Representative images (magnification ×200) were taken from groups of 6 rats.

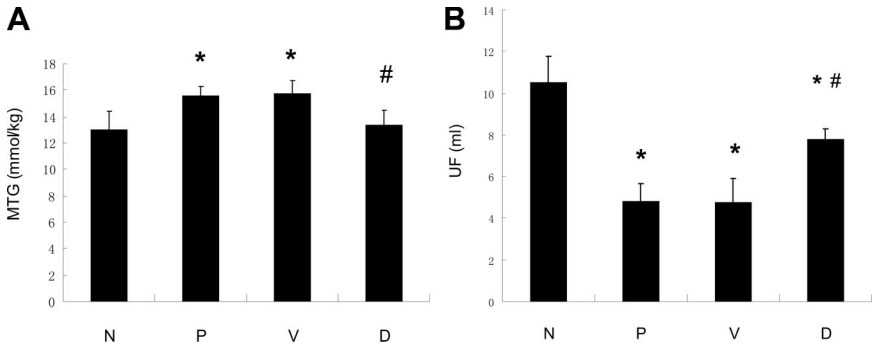

Figure 6. DAPT treatment prevented a loss of peritoneal function caused by PDF. Peritoneal function was assessed by (**A**) mass transfer of glucose (MTG) and (**B**) Ultrafiltration rate (UF). Data are mean ± SD ($n = 6$). $^{*}P < 0.05$ versus normal rats. $^{\#}P < 0.05$ versus PDF treated rats and vehicle control rats. N indicates normal rats; P, PDF-treated rats; V, vehicle control rats treated with DMSO together with PDF. D, Rats treated with DAPT together with PDF.

treatment, and the difference became significant after 10 ng/ml of TGF-β1 treatment compared with control cells. Because the expression of NICD, Jagged-1, and HES-1 reached a plateau at 10 ng/ml of TGF-β1 treatment, we thereof chose the dosage of 10 ng/ml TGF-β1 to study the kinetics of the Notch signaling activation. As shown in Figure 8, C and D, a significant increase of Jagged-1, NICD, and HES-1 was detected, and their levels peaked at 24 hours. The expression of Jagged-1 and HES-1 remained a plateau until 72 hours, whereas the expression of NICD was lower at 72 hours in comparison with that of the 24-hour time point. These results indicated that TGF-β1 could induce Notch signaling activation in a time- and dose-dependent manner in RPMCs.

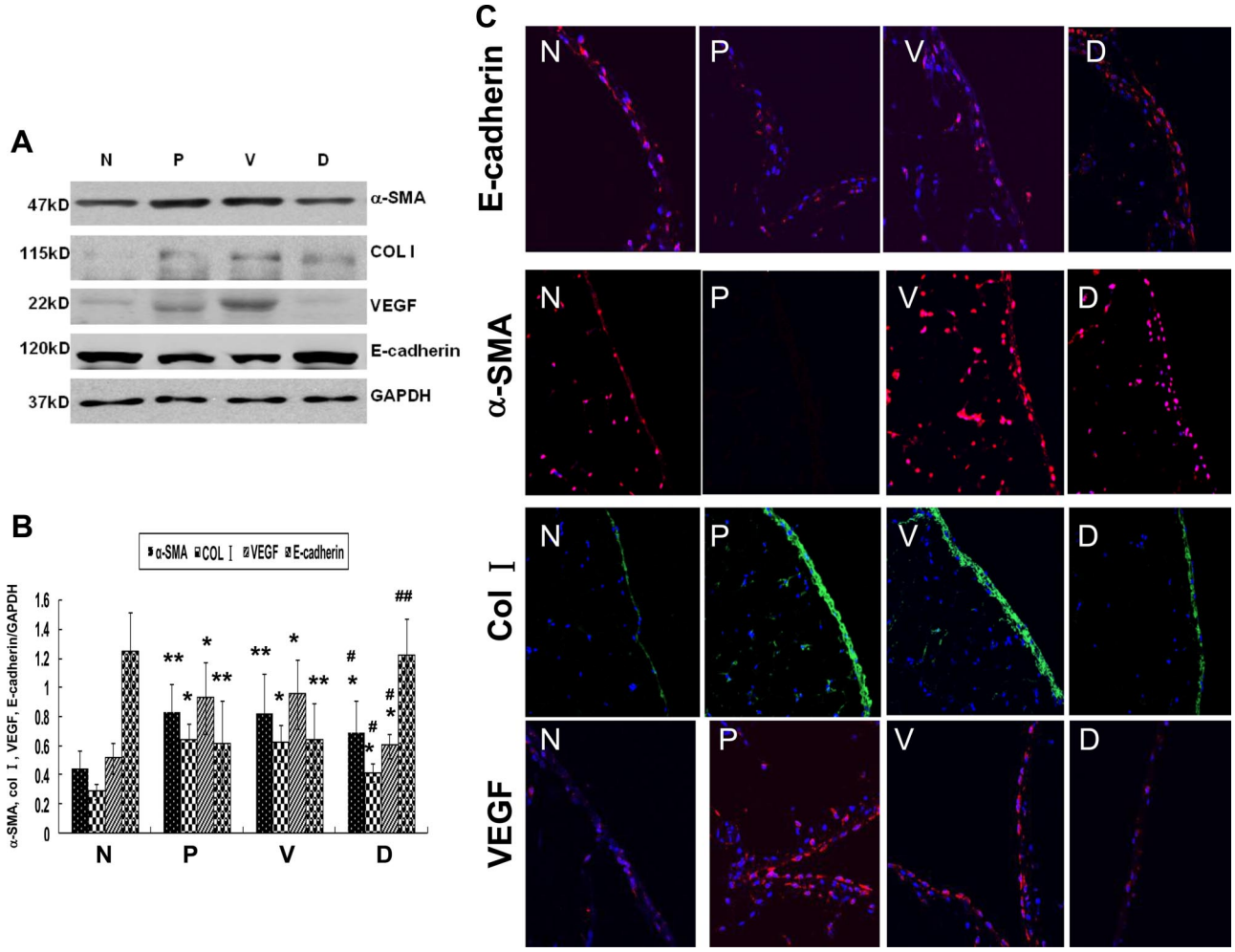

Figure 7. DAPT treatment attenuated the expression of several protein characteristics of EMT and peritoneal fibrosis. **A:** The protein expression of α-SMA, collagen I, VEGF, and E-cadherin in peritoneum was examined by Western blot. **B:** Graphic representation of relative abundance of α-SMA, collagen I, VEGF, and E-cadherin normalized to GAPDH. Data are expressed as mean ± SD ($n = 6$). $^{*}P < 0.05$ versus normal rats. $^{**}P < 0.01$ versus normal rats. $^{\#}P < 0.05$ versus PDF-treated rats and vehicle treated rats. $^{\#\#}P < 0.01$ versus PDF-treated rats and vehicle control rats. **C:** Immunoflurescence staining of α-SMA, collagen I, VEGF, and E-cadherin in peritoneum. Paraffin-fixed peritoneum sections were stained with indicated antibodies. Nuclei were stained with DAPI (blue). Images (magnification ×400) were taken by confocal microscopy. N indicates normal rats; P, PDF-treated rats; V, vehicle control rats treated with DMSO together with PDF. D, Rats treated with DAPT together with PDF.

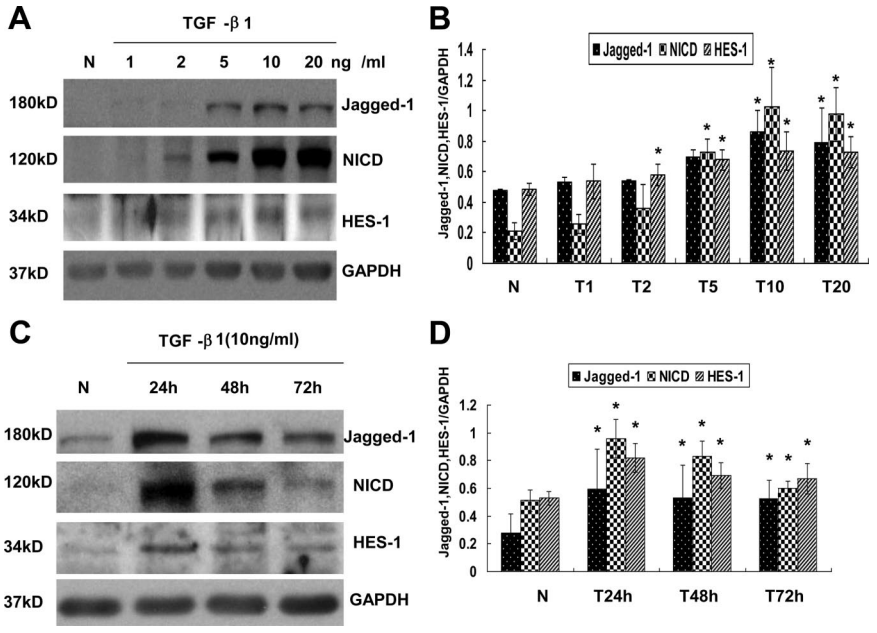

Figure 8. TGF-β1 induced Notch signaling cascade expression in RPMCs in a dose- and time-dependent manner. **A:** RPMCs were treated with the indicated amount of TGF-β1 for 48 hours, and cell lysates were harvested for Western blot analysis with antibodies against NICD, Jagged-1, and HES-1. The membrane was then stripped and blotted with anti-GAPDH to verify equal protein loading. **B:** Graphic representation of relative abundance of NICD, Jagged-1, and HES-1 normalized to GAPDH. Data are given as mean ± SD values of three independent experiments. $^{*}P < 0.05$ versus normal cells. **C:** TGF-β1 induced Notch signaling cascade expression in RPMCs in a time dependent manner. RPMCs were treated with 10 ng/ml of TGF-β1 for the indicated time period, and cell lysates were harvested for Western blot analysis with antibody against NICD, Jagged-1, and HES-1. The membrane was then stripped and blotted with anti-GAPDH to verify equivalent protein loading. **D:** Graphic representation of the relative abundance of NICD, Jagged-1, and HES-1 normalized to GAPDH. Data are given as mean ± SD values of three independent experiments. $^{*}P < 0.05$ versus normal cells.

We next treated RPMCs with DAPT before the addition of TGF-β1. The expression of the Notch downstream target HES-1 was examined by Western blot analyses to monitor the efficiency of DAPT. As shown in Figure 9, A and B, 10-μmol/L DAPT treatment led to a significant decease of HES-1, indicating effective inhibition of TGF-β1–augmented Notch signaling activity. Similarly, TGF-β1–induced EMT of RPMCs was inhibited as indicated by the inhibition of TGF-β1–augmented α-SMA expression, a phenotypic marker of myofibroblasts and a hallmark of EMT at advanced stages. Similarly, DAPT was also effective in the inhibition of ECM accumulation attributable to increased synthesis of ECM proteins such as collagen I. It has been reported that transdifferentiated mesothelial cells synthesize VEGF[6]; therefore, we examined the expression of VEGF in RPMCs. Consistent with previous report, VEGF expression was significantly increased in TGF-β1–treated cells. DAPT treatment significantly attenuated TGF-β1–augmented VEGF expression. Taken together, these data demonstrated that Notch signaling is a downstream of TGF-β, and the key mechanism by which DAPT prevents peritoneal fibrosis is by inhibiting EMT of RPMCs.

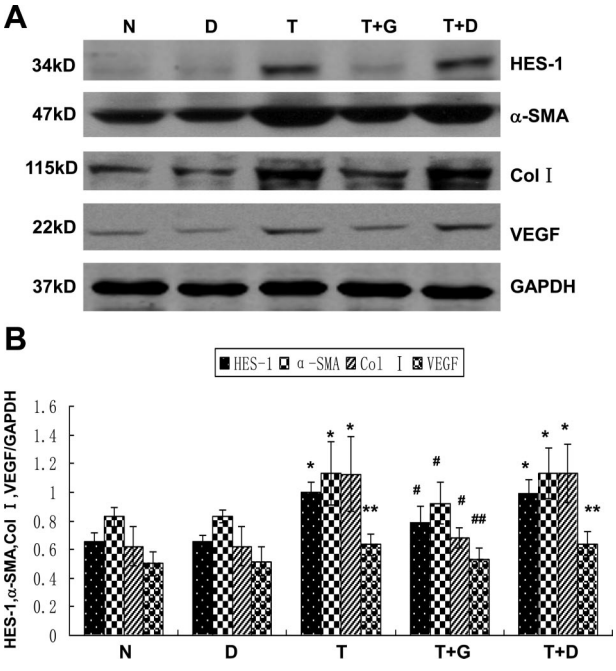

Figure 9. DAPT inhibited TGF-β1–induced EMT of RPMCs. **A:** RPMCs were treated with 10 μmol/L DAPT together with 10 ng/ml TGF-β1 for 48 hours, and cell lysates were harvested for Western blot analysis with antibodies against HES-1, α-SMA, Collagen I, and VEGF. GAPDH was used to verify equivalent loading. **B:** Graphic representation of the relative abundance of α-SMA, Collagen I, VEGF, and HES-1 normalized to GAPDH. Data are given as mean ± SD values of three independent experiments. $^{*}P < 0.05$ versus normal cells. $^{**}P < 0.01$ versus normal cells. $^{\#}P < 0.05$ versus TGF-β1–treated cells. $^{\#\#}P < 0.01$ versus TGF-β1–treated cells. N indicates normal RPMCs; D, RPMCs treated with DMSO; T, RPMCs treated with 10 ng/ml of TGF-β1; T+G, RPMCs treated with 10 μmol/L DAPT plus 10 ng/ml of TGF-β1; T+D, RPMCs treated with DMSO plus 10 ng/ml of TGF-β1.

Discussion

In this study, we showed that Notch signaling was markedly activated in fibrotic peritoneum induced by PDF. This is indicated by the increased expression of NICD, Jagged-1, and HES-1. Likewise, in RPMCs, we detected elevated level of expression for Notch signaling components after TGF-β treatment. Treatment of RPMCs with the Notch inhibitor DAPT led to a strong inhibition of TGF-β–induced EMT. Importantly, DAPT treatment attenuated PDF-induced peritoneum fibrosis *in vivo* as demonstrated by the decreased expression of α-SMA, collagen I, and VEGF. Histochemical analysis further revealed a thinner peritoneum membrane with less ECM accumulation in submesothelial zone after DAPT treatment compared with the PDF-treated group. Importantly, DAPT treatment prevented a loss of peritoneal functions induced by PDF.

Notch signaling is an evolutionarily conserved local cell-signaling mechanism that functions in all metazoa as a major pathway leading to the determination of cellular identity during developmental stages.[38] It has also been reported that Notch signaling is activated in mature organs on injury.[39] Niranjan et al reported that Notch pathway is activated *de novo* in glomeruli (mainly in podocytes) in humans with diabetic nephropathy and FSGS and in rodent models thereof.[40] Kobayash showed an activation of Delta-1/Notch-2/HES-1 pathway in a rat ischemia-reperfusion injury model.[41] In addition, upregulation of Notch-1 and Jagged-1 proteins was detected in the rat liver after partial hepatectomy,[42] and elevated NICD levels was reported in the brain after cerebral ischemi-reperfusion.[43] In this study, our data demonstrated, for the first time, that Notch signaling is elevated during PDF-induced peritoneal fibrosis.

Numerous reports have indicated a role for EMT in fibrosis. Besides TGF-β signaling, Notch signaling pathway was found to contribute to EMT. In endothelial cells, Notch signaling up-regulates Snail to promote mesenchymal transformation, which is critical for normal heart development.[28,44] In human breast epithelial cells, Jagged-1–mediated activation of Notch signaling induces EMT through induction of Slug and subsequent repression of E-cadheirn.[45] During development and tumor progression, Notch seems to be independent of TGF-β signaling. However, several recent studies revealed TGF-β–dependent Notch activation during the process of EMT. A microarray survey of transcriptional changes in human keratinocytes exposed to TGF-β identified increased expression of HES-1.[46] In epithelial cells from mammary gland, kidney tubules, and epidermis, TGF-β induces the Notch target gene *Hey1* at the onset of EMT in a Smad3-dependent manner, followed by delayed Notch-dependent activation of *Hey1*.[30] It has also been reported that Jagged-1 is increased in a TGF-β1–dependent manner in fibrotic renal disease in mice.[29] However, those studies only indicated the role of TGF-β1 on certain component of Notch pathway. In the present study, we observed elevated expression of NICD, Jagged-1, and HES-1 during TGF-β1–induced EMT of RPMCs indicating that Notch signaling pathway is the downstream of TGF-β1. Moreover, increased TGF-β1 expression was detected in rat peritoneum after PDF treatment (data not shown), suggesting that the activation of Notch signaling in fibrotic peritoneum is TGF-β dependent. The mechanism by which TGF-β regulates Notch signaling is currently under investigation. It has been reported that recruitment of Smad3 to CSL, an essential DNA-binding component of Notch signaling, by direct interaction with NICD is responsible for TGF-β–induced HES-1 expression in C2C12 cells.[47] However, our observation that TGF-β induced expression of not only the target gene of Notch signaling, but also Notch receptor and ligand in RPMCs, argues against this scenario.

Besides fibrosis, an increased vascular surface area with a high peritoneal solute transport rate is another cause of ultrafiltration failure after long-term PD.[48–50] VEGF is reported to play an important role in the process that leads to increased vascular permeability by inducing vasodilatation and stimulating angiogenesis.[6,51–53] A number of studies have shown that mesothelial cells have the capacity to produce VEGF in response to a variety of stimuli, and the underlying mechanism of VEGF upregulation in mesothelial cells is the mesenchymal conversion of these cells.[6,54] Consistent with previous studies, we demonstrated an increased expression of VEGF during TGF-β1–induced EMT of RPMCs. The increased VEGF production by mesothelial cells could contribute to the elevated VEGF protein level in whole peritoneum after PDF treatment. Therefore, the effect of DAPT on VEGF expression in peritoneum could be partially explained by its inhibitory effect on EMT of RPMCs. However, we could not exclude the effect of DAPT on the Notch signaling in vascular endothelial cells. Previous studies have demonstrated the expression of Notch-1 and Jagged-1 and Jagged-2 in vascular endothelial cells *in situ*.[55] In addition, activation of Notch signaling with Jagged-1 peptide was reported to enhance vascular endothelial cell proliferation, migration, and tube formation, which are critical for angiogenesis.[39] Based on the results presented herein, we speculate that global targeting of the Notch pathway in peritoneum is a particularly efficient therapeutic strategy because it may serve a dual purpose by inhibiting peritoneal fibrosis and, at the same time, impeding angiogenesis.

Increasing evidence suggests that peritoneal fibrosis is a major cause of failure of PD.[2] Until recently, no efficient treatment for PD-related peritoneal fibrosis is available. To the best of our knowledge, this is the first study to indicate that targeting the Notch signaling, such as by the use of a GSI, may be a novel therapeutic intervention for PD-related peritoneal fibrosis. GSI has been shown to be beneficial in animal models of Alzheimer disease,[56,57] colon cancer,[58] and ischemic stroke.[43] The clinical trial to test the effectiveness of blocking Notch signaling with GSI in T cell acute lymphoblastic leukemia was also initiated.[59] However, the adverse effects of chronic treatment with GSI observed on thymus, spleen, and gastrointestinal hampered this trial.[60] Thus far in our study, intraperitoneal injection of DAPT for 28 days appears to be well tolerated by rats because DAPT treatment did not affect the body weight of rats (data not shown). Jonas Sjölund et al showed that an intermittent administration regimen would allow partial recovery of the small intestine between the successive rounds of drug delivery and thus decreased the adverse effects on the intestine.[61] Notably, Real et al demonstrated that combination therapy with GSI plus glucocortioids delivered by intraperitoneal injection could improve the antileukemic effects of GSI and reduce its gut toxicity in leukemia-bearing mice.[62] These studies provide an effective method to limit the adverse effects of GSI and shed light on the clinical implication of GSI. A further comprehensive evaluation of the optimal administration regime and/or the combined therapy of GSI with glucocortioids may facilitate the clinical development of an optimal GSI-based therapy for PD-related peritoneal fibrosis.

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