MEDI0639: A Novel Therapeutic Antibody Targeting DLL4 Modulates Endothelial Cell Function and Angiogenesis

In Vivo


Abstract

The Notch signaling pathway has been implicated in cell fate determination and differentiation in many tissues. Accumulating evidence points toward a pivotal role in blood vessel formation, and the importance of the Delta-like ligand (DLL4)-Notch1 ligand–receptor interaction has been shown in both physiological and tumor angiogenesis. Disruption of this interaction leads to a reduction in tumor growth as a result of an increase in nonfunctional vasculature leading to poor perfusion of the tumor. MEDI0639 is an investigational human therapeutic antibody that targets DLL4 to inhibit the interaction between DLL4 and Notch1. The antibody cross-reacts to cynomolgus monkey but not mouse species orthologues. In vitro MEDI0639 inhibits the binding of Notch1 to DLL4, interacting via a novel epitope that has not been previously described. Binding to this epitope translates into MEDI0639 reversing Notch1-mediated suppression of human umbilical vein endothelial cell growth in vitro. MEDI0639 administration resulted in stimulation of tubule formation in a three-dimensional (3D) endothelial cell outgrowth assay, a phenotype driven by disruption of the DLL4-Notch signaling axis. In contrast, in a two-dimensional endothelial cell–fibroblast coculture model, MEDI0639 is a potent inhibitor of tubule formation. In vivo, MEDI0639 shows activity in a human endothelial cell angiogenesis assay promoting human vessel formation and reducing the number of vessels with smooth muscle actin-positive mural cells coverage. Collectively, the data show that MEDI0639 is a potent modulator of DLL4-Notch signaling pathway.

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Introduction

The Notch signaling cascade is an evolutionarily conserved pathway that has been implicated in cell fate determination, stem cell maintenance, and differentiation in many tissues during development (1, 2). To date, 4 Notch receptors (Notch1–4) and 5 ligands [Jagged1/2 and Delta-like ligand (DLL) 1/3/4] have been identified in mammals. Notch receptors exist in cell membranes as heterodimers, composed of 2 noncovalently associated extracellular and transmembrane subunits. Receptor–ligand interactions between neighboring cells triggers a series of proteolytic cleavages by enzymes such as TNFα-converting enzyme and γ-secretase, which results in the generation of Notch intracellular domain. The Notch intracellular domain translocates to the nucleus and binds to transcription factors, ultimately causing the activation of downstream target genes, including those of the hairy/enhancer of split (Hes) and Hes-related (Hey) families (3).

An increasing amount of evidence suggests that multiple Notch pathway components are expressed in the vasculature and that aberrations in normal Notch signaling can result in vascular phenotypes. For example, mutations in Jagged1 and Notch3 result in Alagille syndrome and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) respectively, 2 disorders that exhibit vascular defects (4, 5). Furthermore, genetic deletion of either Notch1 or DLL4 in mice both result in embryonic lethality with vascular abnormalities. In addition, deletion of a single allele of DLL4, which is the only Notch ligand expressed predominantly in the endothelium, results in embryonic lethality with severe vascular defects in most genetic backgrounds in mice (6–8). This phenotype has only previously been reported for VEGF-A and suggests...
that Dll4 may play a critical role in vascular development (9, 10).

Dll4 expression has also been reported on the vasculature of several tumor types, including clear-cell renal cell carcinomas, glioblastomas, Ewings sarcoma, and cancers of the breast and bladder (11–15). In addition, expression on a small proportion of tumor cells has also been reported (16, 17). In normal physiology, Dll4-Notch signaling appears to regulate angiogenesis by modulating the number of endothelial tip cells and hence angiogenic sprouting (18). The effect of blocking Dll4 signaling on tumor growth has been evaluated in several preclinical tumor xenograft models of human cancer in which either tumor cell lines or primary human tumors are grown subcutaneously in immunodeficient mice (17, 19, 20), and these studies, reductions in tumor growth have been reported. Agents disrupting Dll4-Notch signaling can have 2 modes of action. First, antagonists can affect survival of tumor-initiating stem cells (17). Second, antagonists increase the number of endothelial tip cells in the tumor, stimulating an angiogenic response, leading to the formation of immature angiogenic vessels. These vessels are not well perfused and poorly functional, reducing the effective blood flow. This increases tumor hypoxia thereby driving a reduction in tumor growth (19–21).

These data suggest that, in addition to its role in vascular development, Dll4 may also play a role in development of the tumor vasculature. In this report, we describe the in vitro and in vivo characterization of a human anti-Dll4-specific monoclonal antibody, MEDI0639, which targets a novel epitope on Dll4 and is a potent modulator of Dll4-Notch signaling.

Materials and Methods
Reagents and cell culture
Recombinant human Notch1-Fc, human Dll4, anti-human Jagged1, anti-human Dll1, and anti-mouse/human Dll4 antibodies were from R&D Systems. Anti-Human IgG-Fc-fluorescein isothiocyanate (FITC), anti-human IgG-Fc-Cy5, and anti-mouse IgG-Fc-Cy5 were from Jackson ImmunoResearch Laboratories, Inc. Anti-human Dll4 antibodies were from R&D Systems. Anti-human Jagged1, anti-human Dll1, and anti-mouse/Jagged1 antibodies were from TCS CellWorks Ltd.

HUVECs were cultured in MCDB131 supplemented with 10% fetal calf serum (FCS) or in endothelial growth media and normal human dermal fibroblasts were cultured in fibroblast growth media. Each vial of primary cells was used for no more than 6 passages. No further authentication was conducted. Cells were maintained in 5% CO₂ at 37°C. Angiogenesis kits and tubule staining kits were obtained from TCS CellWorks Ltd.

Immunization
Immunization protocol is shown in Supplementary Materials and Methods.

Determining binding affinities of MEDI0639
The binding affinities of MEDI0639 were estimated using fluorescence-activated cell-sorting (FACS) techniques. HEK293 cells overexpressing either human or cynomolgus monkey Dll4 were seeded at approximately 85,000 to 104,000 cells/well into 96-well nonadherent plates in PBS and incubated with titrations of purified antibody for 5 hours at 4°C. The cells were then washed and incubated with goat anti-human IgG-Fc-Cy5 + 5 μg/mL 7-aminonacetylmycin (7AAD) for 30 minutes at 4°C. Bound Dll4 was detected using FACS analysis and data were fitted to the following equation:

\[
F = P \left( \frac{K_D + L + n \times M}{2} \right) - \sqrt{\frac{(K_D + L + n \times M)^2 - 4n \times M \times L}{4n \times M \times L}} + B
\]

In this equation, \(F\) = mean fluorescence, \(L_T\) = total molar mAb concentration, \(P\) = proportionality constant that relates arbitrary fluorescence units to bound mAb, \(M\) = cell concentration in molarity, \(n\) = number of receptors per cell, \(B\) = background signal, and \(K_D\) = equilibrium dissociation constant. For each mAb titration curve estimates for \(P\), \(n\), \(B\), and \(K_D\) are obtained by nonlinear analysis fitting of experimental data to the above equation.

Receptor–ligand binding assays
To determine the potency of the panel of antibodies at inhibiting receptor–ligand binding, parental 293T cells or cells transiently transfected with human Dll4 were resuspended in PBS containing 2% FCS and added at a concentration of 25,000 cells/well to a nonadherent 96-well plate containing purified antibodies at a final concentration of 10, 1, or 0.1 μg/mL. After incubation for 1 hour at 4°C, Alexa647–labeled human Notch1-Fc was added at a final concentration of 227 ng/mL and plates were incubated for 2 hours at 4°C. Following washing with PBS containing 2% FCS, the amount of bound Notch1-Fc was determined by reading the fluorescence in each well using a FACSCalibur instrument.

To determine the potency of MEDI0639 at inhibiting binding of Notch1 to human and cynomolgus monkey Dll4, HEK293 cells were used that were stably transfected with either human or cynomolgus monkey Dll4 using retroviral constructs. In these experiments, MEDI0639 diluted in PBS containing 2% FCS was added to Dll4-
expressing HEK293 cells (50,000 cells/well diluted in PBS containing 2% FCS) and incubated for 1 hour at 4°C. Subsequently, Alexa-647-labeled human, Notch1-Fc was added at a final concentration of 0.1 μg/mL and plates were incubated for a further 2 hour at 4°C before washing and reading on a FACSCaliber instrument to determine the amount of Notch-Fc bound.

**Cross-reactivity to human Dll1 and Jagged1**

The 293T cells were mock-transfected or transiently transfected with either human Jagged1 or Dll1. Cells were resuspended in PBS containing 2% FCS and seeded at 50,000 cells/well into a 96-well V-bottomed plate. MEDI0639 diluted in PBS containing 2% FCS was added to the cells and incubated for 1 hour at 4°C. After washing with PBS containing 2% FCS, goat anti-human Fc-Cy5 secondary antibody and 7-AAD were added, and plates were incubated for 15 minutes at 4°C before being washed again with PBS containing 2% FCS and being read on a FACSCaliber instrument. Mouse anti-human Jagged1 antibody detected with anti-mouse Fc-Cy5 secondary antibody or goat anti-human Dll1 antibody detected with anti-goat Fc-Cy5 were used as controls to confirm transfection. Data were analyzed by comparing the shift in geometric mean fluorescence in the mock-transfected cells to that observed in the Jagged1- or Dll1-transfected cells.

**Notch1 cleavage**

Twelve-well tissue culture plates were coated for 4 hours at 37°C with 1 μg/mL of recombinant Dll4 diluted in bicarbonate buffer and then washed twice with PBS. HUVECs were plated at 1.2 × 10^5 cells per well in MCDB131 media + 10% FCS and incubated for 24 hours in the presence of 10 μg/mL of MEDI0639 or isotype control. Protein lysates were prepared in RIPA buffer containing protease inhibitors. Proteins were separated on 4% to 12% bis-TRIS gels and Western blotted with anti-cleaved Notch1 (Val1744) and anti-GAPDH.

**HUVEC proliferation assays**

To prepare Dll4-coated plates, 96-well plates were incubated overnight at 4°C with 100 μL per well of recombinant human Dll4 diluted in bicarbonate buffer. Wells were washed twice with PBS before plating HUVECs. HUVECs were trypsinized and diluted to 4 × 10^4 cells/mL in HUVEC growth media (MCDB131 + 10% FCS). A total of 4,000 cells per well of HUVECs (100 μL/well) were seeded onto Dll4-coated wells or onto plastic. Cells were immediately dosed with 50 μL of prepared test and control antibodies each concentration in triplicate. Cells were incubated at 37°C, 5% CO2 for 96 hours. Proliferation was then determined using the cell counting kit-8 (CCK-8; Dojindo Laboratories). To each well 15 μL of CCK-8 reagent was added and incubated for 4 hours at 37°C, 5% CO2. Absorbance at 450 nm was measured on a Tecan Ultra Microplate Reader.

**Two-dimensional fibroblast-endothelial cells coculture assays**

The effect of Dll4 antibodies on endothelial cell tube formation was measured using both commercially available (TCS CellWorks Ltd.) and in-house angiogenesis kits. For both assays, antibody titrations were performed in MCDB131 media supplemented with 2% FCS. Details of the methods are described in the work of Kendrew and colleagues (22) and shown in the Supplementary Materials and Methods.

**Three-dimensional tubule formation assay**

This assay was run as previously described (22). Detailed method is shown in the Supplementary Materials and Methods.

**Gene expression analysis**

Gene expression analysis was conducted by one-step reverse transcription PCR (RT-PCR). Details of the method and primers are shown in the Supplementary Materials and Methods.

**Epiotpe mapping**

Chimeric variants were engineered with portions of the extracellular domain of Dll4 replaced with the corresponding segments of Dll1. Human Dll4 (ref. 23; cloned in-house) and human Dll1 (accession # NM_005618, OriGene) were used as templates in overlapping extension PCR to construct a series of variants, which include the Dll4 transmembrane domain for surface expression of the recombinant proteins. The resulting variants were cloned into a mammalian expression vector encoding a human cytomegalovirus major immediate early (hCMVie) enhancer, promoter and 5′-untranslated region for transient mammalian expression. The chimeric variants were transiently expressed in HEK293F cells as membrane-bound proteins for flow cytometric characterization with MEDI0639. Forty-eight hours posttransfection, HEK293F transfectants were incubated with 1 μg/mL of MEDI0639 for 1 hour on ice in PBS, washed, then incubated with anti-human IgG-FITC and analyzed with a LSRII flow cytometer. The expression levels of all chimeric variants were monitored by incubating with a mixture of both goat anti-mouse Dll4 (which also recognizes human Dll4) and goat anti-human Dll1 polyclonal antibodies detected with anti-goat IgG-PE.

**Human endothelial cell Matrigel plug model**

This method was as published previously (22). Detailed method is shown in the Supplementary Materials and Methods. Briefly, a mixture of HUVEC spheroids and single endothelial cells were seeded in Matrigel in the presence of VEGF-A and FGF-2 and injected into severe combined immunodeficient (SCID) mice. Vascularization of the plug was assessed by histology after 21 days. For both assays, antibody titrations were prepared in MCDB131 media supplemented with 2% FCS. Details of the methods are described in the work of Kendrew and colleagues (22) and shown in the Supplementary Materials and Methods.

**Results**

MEDI0639 is a potent selective inhibitor of Dll4

The details of the screen for Dll4 antibodies are described in detail in Supplementary Results. Briefly, the
lead antibody MEDI0639 was selected by assessing the ability to modulate the interaction between Dll4 and the Notch1 receptor by FACS, Notch1-Dll4–dependent HUVEC proliferation (19, 24), and endothelial tube formation in a two-dimensional (2D) HUVEC fibroblast cell coculture assay (22, 25), in which inhibition of Notch1-Dll4 signaling inhibits tube formation (Supplementary Fig. S1).

The binding properties of MEDI0639 were characterized by FACS analysis on HEK293 cells expressing different Dll4 species. MEDI0639 showed good binding to human (Fig. 1A) and cynomolgus monkey (Fig. 1B) Dll4 but only weak binding to mouse Dll4 (Fig. 1C). The affinity of MEDI0639 for human and cynomolgus monkey Dll4 was measured by using a cell-based binding assay on HEK293 cells expressing the relevant Dll4. MEDI0639 bound human Dll4 with an affinity of 102 pmol/L and cynomolgus monkey Dll4 with an affinity of 113 pmol/L (Table 1). The potency of MEDI0639 at inhibiting the

Figure 1. Characterization of MEDI0639. A–C, binding of a dose response of MEDI0639 to HEK293 cells expressing human, cynomolgus monkey, or mouse Dll4 was determined by FACS analysis. Data shown are the geometric mean. D and E, inhibition of Notch1-Fc binding to HEK293 cells expressing human or cynomolgus monkey Dll4 was determined by FACS analysis. Data shown are the percent inhibition of Notch1 binding to Dll4 relative to an IgG control. F and G, MEDI0639 does not bind to Dll1 or Jagged. Binding of MEDI0639 to 293T cells transiently expressing either human Dll1 or Jagged1 was determined over a range of doses by FACS analysis. Anti-human Dll1 or anti-human Jagged1 antibodies were used as positive controls. Data were analyzed by comparing the shift in geometric mean fluorescence in the mock-transfected cells to that observed in the Dll1- or Jagged1-transfected cells. Data shown are fold binding compared with an IgG control.
Notch1 interaction with both human and cynomolgus monkey Dll4 was determined using a FACS-based assay with HEK293 cells expressing exogenous human and cynomolgus monkey Dll4. MEDI0639 inhibited the interaction of Notch1-Fc to human Dll4 (Table 1 and Fig. 1D) with a mean EC$_{50}$ of 0.58 ± 0.27 nmol/L (0.086 ± 0.042 µg/mL) and to cynomolgus monkey Dll4 (Table 1 and Fig. 1E) with a mean EC$_{50}$ of 0.44 ± 0.1 nmol/L (0.066 ± 0.015 µg/mL). The weak binding of MEDI0639 to mouse Dll4 was not sufficient to deliver significant inhibition of the interaction between mouse Notch1 and Dll4 (data not shown).

Table 1. Potency and affinity of MEDI0639

<table>
<thead>
<tr>
<th>Property</th>
<th>Human</th>
<th>Cyno</th>
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<tbody>
<tr>
<td>Receptor ligand inhibition on cells (EC$_{50}$ nmol/L ± SD)</td>
<td>0.58 ± 0.27 (n = 8)</td>
<td>0.44 ± 0.1 (n = 3)</td>
</tr>
<tr>
<td>Affinity to cells [K$_D$ pmol/L (95% CI)]</td>
<td>102 (49.7–155)</td>
<td>113 (94.6–131)</td>
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NOTE: Inhibition of Alexa-647–labeled human Notch-Fc binding to HEK293 cells stably expressing human or cynomolgus monkey Dll4 by FACS analysis. Affinity of MEDI0639 to HEK293 cells stably expressing human or cynomolgus monkey Dll4.

Abbreviation: CI, confidence interval.
MEDI0639 Targets Dll4

Figure 3. MEDI0639 effect in a 2D fibroblast endothelial cell coculture assay. A, MEDI0639 inhibits tube formation in a 2D tube assay in a dose-dependent manner. Fibroblast and HUVEC cocultures were treated with a dose response of MEDI0639 or with 20 µg/mL of isotype control for 11 days. Data shown are quantification of CD31-positive vessel length (mm). B, images of 2D tube assay treated with a dose response of MEDI0639. Day 11 cultures were fixed and tubules visualized by staining for CD31. Data shown are images taken at ×5 magnification. C, effect of MEDI0639 on 2D tube formation over time. Fibroblast and HUVEC cocultures were treated with 10 µg/mL of MEDI0639 or an isotype control for 10 days. Data shown are the relative tube count × tube length on days 2, 4, 7, and 10 of the assay. D, images of 2D tube assay treated with 10 µg/mL of MEDI0639 or isotype control. Cultures were fixed on days 2, 4, 7, and 10 and tubules visualized by staining for CD31. Data shown are images taken at ×5 magnification. E–G, effect of MEDI0639 on the expression of CD31, HEY1, and APLN in the 2D tube formation assay. Expression of CD31, HEY1, and APLN on days 2, 4, 7, and 10 of the tube assay were determined by quantitative RT-PCR. Data shown are expression of CD31 relative to 18S and expression of HEY1 and APLN relative to CD31.

The selectivity of MEDI0639 for Dll4 versus its most closely related family members, Dll1 and Jagged1, was determined using a cell-based binding assay. MEDI0639 did not bind to 293T cells expressing either Dll1 (Fig. 1F) or Jagged1 (Fig. 1G) at concentrations up to 300 µg/mL. Antibodies to either Dll1 or Jagged1 confirmed expression of the ligand on the cells (Fig. 1F and G).

MEDI0639 modulates endothelial cell function in vitro

Engagement of HUVECs with Dll4 causes the cleavage of the Notch1 receptor (26), therefore an antibody targeting the Dll4 ligand would be expected to inhibit the cleavage. MEDI0639 completely inhibited the Dll4-induced cleavage of endogenous Notch1 expressed on HUVECs (Fig. 2A). The potency of MEDI0639 at reversing the Dll4 suppression of HUVEC proliferation was determined. MEDI0639 completely reversed Dll4 suppression of HUVEC proliferation down to 2.2 µg/mL (Fig. 2B).

To assess the ability of MEDI0639 to promote tube formation, a three-dimensional (3D) coculture assay was used. Beads coated with endothelial cells and fibroblast cells were cocultured in a fibrinogen matrix (22). In this assay, endothelial tubules grow out from the bead mimicking the early phases of angiogenesis. Consistent with the mechanism of action of a potent inhibitor of Dll4-Notch1 signaling, addition of MEDI0639 at 10 µg/mL resulted in substantial stimulation of tube formation in the presence of VEGF-A, an effect of tube formation was not evident at 1 µg/mL (Fig. 2C and D).

To determine the potency of MEDI0639 in an endothelial cell culture assay, we used a 2D fibroblast–endothelial cell coculture assay. This assay differs from the 3D outgrowth assay in that single endothelial cells form multicellular endothelial tubules supported by a fibroblast feeder layer. Tubules form as a result of single cell associating to form tubules, rather than tubule outgrowth. The advantage of this assay is that it can be readily quantified using standard imaging techniques. In the 2D tube assay, MEDI0639 fully inhibited tube formation down to 2.2 µg/mL and activity was lost at 0.03 µg/mL (Fig. 3A and B). Commonly antagonism of the Dll4-Notch1
signaling pathway results in increased angiogenesis both in vitro and in vivo (19–21). To investigate the effects in the 2D assay in more detail we studied a time course of tube formation in the presence of MEDI0639. MEDI0639 reduced tube formation relative to control as early as 2 days (Fig. 3C and D). The changes in expression of specific endothelial genes by MEDI0639 at each time point were studied by quantitative RT-PCR (Fig. 3E–G). Consistent with the visualized tubules, a decrease in CD31 gene expression relative to total 18s was detected, suggesting a reduction in the relative number of endothelial cells in the MEDI0639-treated wells relative to IgG control over time. In addition, the Notch-regulated gene HEY1 was reduced and the endothelial tip cell gene APLN (27) was increased (Fig. 3F and G). Consistent with MEDI0639 reducing Notch pathway activation and promoting tip cell formation, the reduction in HEY1 and concomitant induction of APLN was clearly observed by day 4. These data confirm that MEDI0639 modulates endothelial signaling through the Notch pathway and promotes the formation of tip cells.

Epitope mapping of MEDI0639

MEDI0639 binds specifically to human Dll4 but does not recognize human Dll1 (Figs. 1F and 4B). This specificity was used to determine the binding epitope of MEDI0639 to human Dll4. Eighteen chimeric variants were constructed by swapping in or out various domains of the extracellular domain of Dll1 into Dll4 (KO, knockout variants) or of Dll4 into Dll1 (KI, knock-in variants; Fig. 4A). All variants encoded a transmembrane domain for cell-surface expression of the chimeric protein. The binding characteristics of MEDI0639 to these variants were analyzed by flow cytometry. All variants expressed well as monitored by anti-Dll4 and anti-Dll1 polyclonal antibodies (Fig. 4B and C). MEDI0639 bound well to all of the KO or KI variants encoding for either the DSL or EGF1 domain of Dll4 (KO_N-ter1, KO_N-ter2, KO_N-ter, KO_DSL, KO_EGF1, KO_EGF2, KO_EGF34, KO_EGF5678, KO_N-ter+DSL, KO_DSL, KO_EGF1, and KO_DSL+EGF1; Fig. 4B). However, MEDI0639 did not recognize any of the KO variants that encoded both the DSL and EGF1 domains of Dll1 (KO_DSL+EGF1, KO_DSL+EGF12, and KO_N-ter+DSL+EGF12; Fig. 4B). Thus, the DSL and EGF1 domains of Dll4 contain the binding epitope of MEDI0639.

To further refine the binding epitope of MEDI0639 within the DSL and EGF1 domain of Dll4, 3 additional chimeric variants were constructed. Three, 15 amino acids segments within the DSL and/or EGF1 domains of Dll4 were substituted with the corresponding Dll1 amino acids.
in vivo

DLL4, this was insufficient to give the level of functional
as the epitope for binding of MEDI0639 and identifies 15
prevented binding of MEDI0639 (Fig. 4C; KO_A 187-201).

KO_A, KO_B, KO_C; Fig. 4A). No effect on MEDI0639
binding was observed when amino acids 200 to 214 or 210
to 224 of DLL4 were replaced (Fig. 4C; KO_B 200-214 and
KO_C 210-224, amino acid numbering corresponding to
mature protein). However, substitution of amino acids
187 to 201 of DLL4, which span both the DSL and EGF1
domains, with the corresponding amino acids in DLL1
prevented binding of MEDI0639 (Fig. 4C; KO_A 187-201).

These data show that the DSL and EGF1 domains of DLL4
as the epitope for binding of MEDI0639 and identifies 15
amino acids (187-201) as critical for binding.

MEDI0639 modulates angiogenesis in vivo

Although MEDI0639 showed weak binding to mouse
DLL4, this was insufficient to give the level of functional
blockade required to model MEDI0639 in vivo using
standard models. Therefore, to show that MEDI0639 was
able to modulate angiogenesis in vivo, we used a human
endothelial cell Matrigel plug model in which human
endothelial cells are seeded into Matrigel and implanted
into SCID mice in the presence of VEGF-A and FGF-2 to
form fully functional human vessels (28). We previously
used this model to show in vivo activity for a VEGFR-2
inhibitory antibody (22). Treatment with MEDI0639 at 1
mg/kg resulted in a 3-fold induction in vessel number
(Fig. 5A and D). This was associated with a concomitant
reduction in the percentage of smooth muscle actin
(SMA)-positive mural cells associated vessels within the
plug, consistent with MEDI0639 stimulating a proangiogenic
response and the formation of increased numbers of
neangiogenic vessels (Fig. 5B). In contrast, inhibition of
VEGFR-2 with the inhibitory antibody 33C3 resulted in a
significant reduction in the vessel number. The proangiogenic
effect of MEDI0639 was not apparent when dosed at

Figure 4. (Continued) B, FACS analysis of binding of MEDI0639 to chimeric DLL4/1 variants transiently expressed on the surface of HEK293F cells. Expression of the DLL4/1 variants was monitored using polyclonal anti-DLL4 and anti-DLL1 antibodies. MEDI0639 did not bind to any of the chimeric variants encoding for the DSL and EGF1 domains of DLL1 (KO_DSL + EGF1, KO_DSL + EGF1, and KO_N-ter + DSL + EGF1). C, fifteen amino acids (187-201) are critical for MEDI0639 specificity. Chimeric knockout (KO) variants encode DLL4 with segments of the DLL and EGF1 domains substituted with the corresponding regions of DLL1. The expression levels of the variant proteins were monitored with 2 combined anti-DLL4 and anti-DLL1 antibodies. Substituting segment A (aa 187–201) in the junction between the DSL and the EGF1 domains of DLL4 with the counterpart of DLL1 (KO_A) resulted in the loss of binding of MEDI0639.
0.02 mg/kg. When the dose response was examined, MEDI0639 was able to stimulate significant increase in vessel number at doses as low as 0.04 mg/kg, with significant effects lost at 0.02 mg/kg (Fig. 5C). These data establish that MEDI0639 is able to modulate the Dll4-Notch1 interaction in vivo resulting in a significant stimulation of neoangiogenic vessels.

Discussion

Emerging evidence suggests that multiple members of the Notch pathway are expressed in the vasculature and that aberrations in normal Notch signaling can result in modulation of vascular phenotypes (18, 29). Here, we report the discovery of MEDI0639, a novel human antibody that targets the Notch ligand, Dll4. MEDI0639 binds with high affinity to both human and cynomolgus monkey Dll4 and inhibited Dll4 function but showed only weak binding to mouse Dll4. MEDI0639 binds a novel epitope on DLL4, localized within the DSL and EGF1 domains. This epitope appears to be differentiated from other functional anti-Dll4 antibodies (17, 19).

The functional consequences of MEDI0639 administration were evaluated in 2 different endothelial cell–fibroblast cell coculture assays. First, MEDI0639 was evaluated in a modified 3D spheroid-based angiogenesis model in 0.02 mg/kg. When the dose response was examined, MEDI0639 was able to stimulate significant increase in vessel number at doses as low as 0.04 mg/kg, with significant effects lost at 0.02 mg/kg (Fig. 5C). These data establish that MEDI0639 is able to modulate the Dll4-Notch1 interaction in vivo resulting in a significant stimulation of neoangiogenic vessels.

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The functional consequences of MEDI0639 administration were evaluated in 2 different endothelial cell–fibroblast cell coculture assays. First, MEDI0639 was evaluated in a modified 3D spheroid-based angiogenesis model in
which HUVECs are grown in fibrin gels in the presence of fibroblasts (22). In the presence of VEGF-A, MEDI0639 was capable of inducing endothelial cell sprouting. This effect is consistent with previously published studies that highlight the role of Dll4 as a negative regulator of angiogenesis and the phenotypic consequence of inhibition being an increase in vessel branching due to an increase in the number of tip cells (18, 19, 30). Second, MEDI0639 was evaluated in a 2D coculture assay, in which endothelial tubes form on top of a fibroblast monolayer. In the 2D coculture assay, MEDI0639 was a potent inhibitor of both vessel length and the number of bifurcations. The ability to cause this phenotypic change was not a unique property of MEDI0639, as it was also observed with additional antagonistic anti-Dll4 antibodies, as well as other Notch pathway antagonists, including γ-secretase inhibitors and soluble Notch receptors. The difference in phenotypes observed serve to further highlight the contextual significance of Notch signaling in angiogenesis and may reflect that in the 2D coculture assay, the inhibition of the Notch pathway helps to promote or maintain endothelial cells in a tip cell phenotype and hence prevent differentiation and organization into tubules. Therefore, the phenotype observed in the 2D assay is consistent with MEDI0639 promoting the formation of individual tip cells and preventing formation of stalk cells, as evidenced by the increase in expression of the endothelial tip cell marker APLN (27) whereas reduction in HEY1 expression confirms that this is related to the inhibition of Notch signaling. These changes in expression are consistent with modulation of the phenotype of the endothelial cells. Insights into Dll4 signaling using in vitro systems have been investigated using assays that rely on either overexpression of exogenous Dll4 or Notch1 and or stimulation with recombinant protein (31, 32). The 2D coculture assay provides a system where the primary signaling events driven by endogenous Dll4-Notch1 cell–cell signaling can be studied.

As the antibodies were not effective functional inhibitors of mouse Dll4, activity in vivo was evaluated using a mouse model in which human endothelial cells are engrafted as spheroids in mice (33). In the absence of treatment, the engrafted HUVECs mature and connect to the mouse vascular system to establish a functional and pericyte-covered vasculature. However, in the presence of MEDI0639, dose-dependent increases in vessel formation were observed, concomitant with a striking decrease in mural cell coverage, as determined by the percentage of CD34-positive endothelial cells that were associated with α-SMA–expressing cells. While the role of Dll4 signaling in endothelial cell proliferation has been the subject of much exploration, the role of Dll4 in the regulation of vessel maturation and pericyte recruitment/formation during vessel development is less established. In surviving Dll4 heterozygous animals, both similar and lower levels of pericyte coverage relative to wild-type controls have been reported (18, 21). In addition, suggestive of a role for Dll4 in vessel maturation, in bladder cancer, a correlation between Dll4 expression and vessel pericyte coverage has been reported (14). Furthermore, decreases in pericyte coverage have recently been reported in studies in which Dll4 has been overexpressed in, for example, human U87 tumor cells before in vivo transplantation (34). These decreases were shown to be concomitant with increased Notch signaling in the tumor stroma. Collectively, these data suggest a critical dosage sensitivity requirement for Notch signaling in angiogenesis and that perturbations in either direction are deleterious to the overall process. In keeping with our data, recent studies in Ewings sarcoma xenograft models report that blockade of Dll4 using either shRNA or a mouse cross-reactive anti-Dll4 antibody is associated with a decrease in pericyte/vSMC coverage (13, 35). Taken together, our findings are consistent with a role for Dll4-Notch signaling in pericyte/vSMC development and, in future studies, it will be interesting to further elucidate the role of Dll4 relative to other Notch pathway members (e.g., Notch3) in pericyte recruitment and vessel maturation. Our data also provide additional mechanistic understanding to previous reports that have shown positive combination activity of dual VEGF and Dll4 signaling blockade in several tumor xenograft models (19), with Dll4 inhibition potentially increasing vessel sensitivity to anti-VEGF–based agents through the maintenance of an immature vasculature. Moreover, the data show the use of the human endothelial cell Matrigel model to study the mode of action of vascular-modulating agents, as the results obtained phenocopy those seen in human xenograft models.

Preclinical data suggest that Dll4 antagonists have potential as therapeutics to target tumor vasculature in a different way from classic antiangiogenic drugs such as VEGF antagonists, and therefore may have different clinical use. Although not assessed in the current study these agents have the potential to target tumor-initiating cells (17). Agents such as MEDI0639 can be used to test these concepts both preclinically and in the clinical setting. Moreover, agents targeting different epitopes in these key receptors may deliver different outcomes. In summary, this work describes the discovery of novel human monoclonal antibodies directed against Dll4 and provides additional preclinical data to support the role of Dll4 as an important regulator of angiogenesis and vessel maturation. Taken together, these data further support the continued development of anti-Dll4–neutralizing agents as single agent or combination treatments for human cancers or in other settings dependent upon angiogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: D.W. Jenkins, D.C. Blakey, V. Bedian, S.T. Barry
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Ross, M. Veldman-Jones, J. Kendrew, P. Petteruti, L. Peng
References

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