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Comparison of doxycycline and minocycline in the inhibition of VEGF-induced smooth muscle cell migration

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Abstract

Smooth muscle migration plays an important role during angiogenesis and vascular remodeling. In this study, we examined the effects of doxycycline and minocycline on vascular endothelial growth factor (VEGF)-induced human aortic smooth muscle cell (HASMCs) migration, and explored the mechanisms in which doxycycline or minocycline inhibit HASMC migration. We demonstrated that both doxycycline and minocycline attain consistent anti-angiogenic effects in the inhibition of HASMC migration via a different signal pathway ($p < 0.05$). This effect is through attenuating VEGF-induced matrix metalloproteinase-9 (MMP-9) activity ($p < 0.05$). Doxycycline could increase tissue inhibitors of metalloproteinases-1 (TIMP-1) expression while minocycline down-regulated PI3K/Akt phosphorylation in HASMC. Our study suggests that doxycycline has a stronger ability to inhibit MMP secretion in HASMC by up-regulating endogenous MMPs inhibitor TIMP-1, while minocycline implements anti-angiogenic effect through inhibiting HASMC migration by down-regulating PI3K/Akt pathway.

Keywords

Doxycycline; Matrix metalloproteinase; Minocycline; Migration; Smooth muscle cell; Vascular endothelial growth factor

1. Introduction

Smooth muscle cell (SMC) migration plays an important role in angiogenesis and disease-related microvascular remodeling. SMC migration can be found in several pathological conditions such as brain arteriovenous malformations, pulmonary hypertension, arteriosclerosis and restenosis after angioplasty (Fanburg et al., 1997; Owens, 1995; Ross, 1995; Stenmark et al., 1997). VEGF plays an important role in these processes since VEGF receptors can be detected in the SMCs (Goncharova et al., 2002; Grosskreutz et al., 1999). Cerebrovascular diseases, such as aneurysms, ischemia, and hemorrhage, up-regulate VEGF expression (Hashimoto et al., 2005; Hayashi et al., 2003). Therefore, we chose VEGF as a

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stimulator to study the effect of doxycycline and minocycline during human aortic SMC migration *in vitro*.

Doxycycline and minocycline are second-generation tetracyclines, and remain widely used as antibiotics. Current studies have demonstrated that they are beneficial drugs affecting many mammalian cell functions, including cell proliferation, migration, and matrix remodeling *in vitro* (Nelson, 1998). Doxycycline and minocycline also show anti-angiogenesis activities *in vivo* (Lee et al., 2004). Recent studies demonstrate that doxycycline and minocycline affect many cellular functions, and that these biological effects are completely separate and distinct from its anti-microbial action (Ryan et al., 1996). For example, doxycycline inhibits the activity of collagenase, gelatinase and stromelysin (Gilbertson-Beadling et al., 1995; Golub et al., 1991), and therefore has been used to reduce tissue degradation in aortic aneurysms and arthritis, and inhibit tumor cell invasion and metastasis (Fife et al., 1995; Seftor et al., 1998; Tamargo et al., 1991). Doxycycline and minocycline inhibit human umbilical vascular endothelial cell proliferation and tube formation, tumor cell proliferation and migration, and inducible nitric oxide synthetase expression *in vitro* (Bettany et al., 1998; Fife et al., 1997; Fife et al., 2000), and also impact many of these processes *in vivo*. Doxycycline inhibits outward vessel remodeling in response to increased blood flow (Ramamurthy et al., 1993; Tronc et al., 2000), and improves function in isolated rat heart injury by ischemia-reperfusion (Meier et al., 1999). In balloon catheter denudation of the rat carotid artery, doxycycline inhibits SMC migration through the reduction of MMP-2 and MMP-9 activity in the arterial wall (Bendeck et al., 2002). Although doxycycline and minocycline are functionally highly similar, it is unclear if they share the same signal pathway in VEGF-induced HASMC migration.

The present study aimed to compare the mechanism in the inhibition of VEGF-induced HASMC migration between doxycycline and minocycline through their signal pathway.

2. Materials and methods

2.1. Materials

HASMCs were purchased from Cell Systems (St. Katharinen, Germany), and their homogenous nature was confirmed through immunostaining with anti-SM- α -actin (Chemicon, Temecula, CA). Fetal bovine serum and Trizol reagent were obtained from GIBCO (Grand Island, NY), doxycycline from Sigma (St. Louis, MO), VEGF from R&D Systems (Minneapolis, MN), zymographic standards from Chemicon (Temecula, CA), and Coomassie Brilliant Blue from Bio-Rad (Richmond, CA). The RETROscript kit was from Ambion (Austin, TX), and the hybond nitrocellulose membrane from Amersham (Piscataway, NJ). MMP-9 Oligonucleotide primers and fluorescent-labeled TaqMan probes were designed using Primer Express 1.0 software from Applied Biosystems (Foster City, CA). TIMP-1 was obtained from New Marker (Fremont, CA), and Phosph-Akt from Cell Signaling (Beverly, MA). Kodak film came from the Eastman Kodak Company (Rochester, NY).

2.2. Human aortic smooth muscle cell culture

HASMCs were incubated in DME medium with 10% fetal bovine serum and maintained at 37°C in 5% CO₂/95% ambient mixed air. The culture media was changed every 2 days. HASMC experiments were performed on 5 to 8 passages. During the VEGF, doxycycline and minocycline treatment experiments, HASMCs were grown in 10% FBS in 35 mm polystyrene plates to 80% confluence and rinsed 3 times with a serum-free medium, then arrested with 0.2% BSA for 24 hours. Since we found doxycycline and minocycline treatment at the concentration of 10–30 μ M without VEGF did not affect cell phenotypes including migration

(data not shown), HASMCs were incubated with doxycycline and minocycline and then treated with VEGF.

2.3. Human aortic smooth muscle cell invasion assay

HASMC migration was evaluated using 24-well Transwell cell culture chambers with 8.0 μ m pore polycarbonate filter inserts. The filters were coated with matrigel (Pauly et al., 1994; Wang et al., 1998). The stock solution of matrigel was diluted to 300 μ g/ml using serum-free DME medium. An aliquot of 75 μ l matrigel was added into each filter insert and incubated overnight at room temperature under a laminar flow hood. The next day, the coated insert was rehydrated with 0.5 ml of serum-free DME medium for 2 hours. Cultured HASMCs were trypsinized and suspended in 0.2% BSA/DMEM at a concentration of 2.5×10^5 /ml. A total of 500 μ l of 0.2% BSA/DMEM was added to the lower chamber and 100 μ l cell suspension was applied to coated insert filters. The chamber was incubated at 37°C/5% CO₂ for 1 hour to allow cell attachment. HASMCs were treated with doxycycline (0–100 μ M) and minocycline (0–120 μ M) at increasing concentrations. A concentration of 20 ng/ml of VEGF was added to the lower compartment of each chamber. Negative control was treated by 0.2% BSA/DMEM and positive control was treated by 30 μ M of PD98059, which we used in a previous study (Yao et al., 2004). The chamber was incubated for 18 hours at 37°C/5% CO₂ to allow cell migration; the insert was then removed and the membrane washed with 0.1M PBS. Unmigrated cells on the upper side of filter were scraped; migrated cells on the lower side of chamber were fixed and stained with hematoxylin. The membrane was mounted on a slide and then examined under a microscope using 10X magnification. Migration was quantified by measuring the stained cells in five random areas per membrane.

2.4. MMP-2, -9 zymograms

Zymograms are electrophoresis gels with embedded gelatin (Rosenberg et al., 1994), and were used to detect MMP-2, -9 activity (Southgate et al., 1992). The cultured HASMCs grew to 80% confluence in 35 mm plates (concentration of 2.5×10^5 /ml cells) in a 10% FBS media. These cells were incubated in serum-free medium for 24 hours after removing 10% FBS medium. HASMCs were then treated with minocycline and with/without VEGF (20 ng/ml) at the same time for 24 hours. Aliquots of conditional medium (containing ≈ 50 μ g protein) were mixed with 2X sample buffer and loaded on a 10% polyacrylamide gel incorporated with 0.1% gelatin for electrophoresis. MMP-2, -9 zymographic standards were used as the standard. Gels were denatured for 30 minutes, developed overnight, stained with 0.25% Coomassie brilliant blue, and destained to visualize the MMP-2, -9 bands.

2.5. Real time RT-PCR for MMP-2 and MMP-9

Cytoplasmic RNA was extracted from cultured cells using Trizol reagent, and RT-PCR was performed using a RETROscript kit. The first-strand cDNAs were synthesized from 1 μ g total RNA using 50 U MuLV reverse transcriptase, 20 U RNase inhibitor and 50 pmol oligo (dT) as a primer at 50°C for 45 minutes and denatured at 95°C for 2 minutes. PCR reaction for MMP-9 was performed 36 cycles at 95°C for 1 minute, 58°C for 1 minute, and 68°C for 7 minutes with the following primers: Forward: 5'-CGCTCAGATCCGTGGTGAG-3' and Reverse 5'-TGTCACGTGGCGTCACAGT-3' for MMP-2 and 5'-6-FAM d (TTCTTCTTCAAGGACCGTTCATTTGGC) BHQ-1 3 for GAPDH; Forward: 5'-CCGCAGGGCCCCTTCCTTAT-3' and Reverse 5'-GCCCACTTGGTCCACCTGGTT-3' for MMP-9 (amplified length, 216 bp) and 5'-GCCAAGGTCATCCATGACAAC-3' and 5'-GTCCACCACCCTGTTGCTGTA-3' for GAPDH (amplified length, 498 bp). Amplified product (10 μ l) was separated through 1.5% agarose gels and visualized on an UV

transilluminator. Relative MMP-9 mRNA expression was normalized by GAPDH and quantified as fold increase compared with control, as measured by optical density.

2.6. Phospho-Akt and TIMP-1 expression

Phospho-Akt, and TIMP-1, -2 expression were semi-quantified by Western blot analysis. HASMCs grew to approximately 80% confluence, and made quiescent with serum-free medium for 24 hours. After being pretreated with doxycycline or minocycline, VEGF was added. Plates were then incubated 20 min for phospho-PI3K/Akt and 24 hours for TIMP-1. Conditional mediums were collected and concentrated to measure TIMP-1, -2 expression. Cells were washed twice with PBS and scraped into a lysis buffer to detect the PI3K/Akt. Equal amounts of protein (50 µg/lane for TIMP-1, 20 µg/lane for PI3K/Akt) were electrophoresed through a 10% SDS-PAGE gel, and blotted onto a hybond nitrocellulose membrane. The membrane was blocked by 5% non-fat milk solution in TBS/0.1% Tween-20 for 1 hour at room temperature, and then incubated with anti-TIMP-1/2 (both in 1:100 dilution), or rabbit polyclonal p-PI3K/Akt antibodies overnight; secondary HRP-labeled anti-rabbit IgG antibody was added for 1 hour. The membrane was plastic-wrapped and exposed to Kodak film. Bands were scanned and semi-quantified by densitometry. After scanning, the membrane was stripped and re-incubated with β-actin for the internal control.

2.7. Smooth Muscle Cell Viability

The cytotoxicities of doxycycline and minocycline on HASMCs were determined using a MTT assay (Lokeshwar et al., 2002). HASMCs were grown in 96-well microtiter plates with 100 µl of culture medium for 24 hours. Then the mediums were removed, and HASMCs were treated with doxycycline and minocycline at increasing concentrations in 0.2% BSA/DMEM. The culture plates were incubated at 37°C/5% CO₂ for 30 minutes and 20 ng/ml VEGF was added. The plates were then incubated for 36 hours and the medium was replaced with 100 µl of fresh DMEM containing 0.2% BSA. Next, 2mg/ml MTT solution was added and the plates were incubated again for 3 hours at 37°C/5%CO₂. Finally, the medium containing MTT was aspirated off and 150 µl of DMSO solution was added. As a result, living cells formed crystals due to the presence of MTT. Crystals were solvated by DMSO and the absorbance was measured at 570 nm using an ELISA reader.

2.8. Statistical analysis

Results of the dose-dependent migration and inhibition experiments were analyzed using an ANOVA and post-hoc multiple comparison test. All data are represented as mean±standard deviation (mean±SD). A random probability value less than 0.05 is considered statistically significant for the comparisons.

3. Results

3.1. Effect of doxycycline/minocycline on VEGF-induced SMC invasion into extracellular matrix

Biological activity associated with MMP up-regulation in HASMCs was examined using a migration assay with modified Boyden chamber. We established that VEGF enhances HASMC invasion into matrix barriers in a dose dependent manner (Yang et al., 2004). Here, we treated HASMCs with doxycycline and minocycline. We found that both doxycycline and minocycline inhibit HASMC migration in a dose-dependent manner (Figure 1). Doxycycline at 10 and 20 µM and minocycline at 15 and 30 µM reduced HASMC migration to 17–35% and 17–46% of the control group (VEGF alone), respectively ($p<0.05$).

3.2. Doxycycline and minocycline inhibit MMP-9 activity in VEGF-treated HASMCs

Doxycycline or minocycline at 60 μ M concentration were not cytotoxic to confluent HASMCs. Doxycycline and minocycline at 100 and 120 μ M resulted in a 40% reduction of viability (Figure 2), suggesting that the dose range in our experiments is safe, and that the effects of doxycycline and minocycline on HASMC migration and MMP activity are not due to the cytotoxicity caused by doxycycline and minocycline.

Further analysis demonstrated that both doxycycline and minocycline could inhibit MMP-9 latent and active forms in a dose dependent manner (Figure 3, $p < 0.05$). Minocycline was more efficient in inhibiting MMP-9 activity compared to doxycycline. In addition, high doses of minocycline and doxycycline also inhibited latent MMP-2 form in the VEGF-treated HASMCs (Figure 3, $p < 0.05$). To further confirm the effects of minocycline and doxycycline on MMP-2, we examined MMP-2 at mRNA level. Our result demonstrated that both minocycline and doxycycline did not affect MMP-2 mRNA expression (Figure 4).

3.3. Doxycycline and minocycline inhibit MMP-2, -9 mRNA expression

We verified that VEGF-stimulated MMP-9 mRNA overexpression in the HASMCs with a dose-dependent manner (Figure 5), while VEGF did not up-regulate MMP-2 mRNA expression in the HASMCs ($p > 0.05$). The study also demonstrated that doxycycline and minocycline at 15 μ M concentration could inhibit VEGF-induced MMP-9 mRNA but not MMP-2 mRNA expression ($p < 0.05$).

3.4. Minocycline but not doxycycline inhibits PI3K/Akt signal in VEGF-treated HASMCs

We found that minocycline (but not doxycycline) could inhibit ERK1/2 pathways (Lee et al., 2006). We next explored whether doxycycline and minocycline regulated VEGF-induced HASMC migration through PI3K/Akt signaling pathway. Phosphorylation of PI3K/Akt signaling was upregulated in the VEGF-treated HASMCs. Minocycline (but not doxycycline) could inhibit PI3K/Akt phosphorylation ($p < 0.05$, Figure 6). The inhibiting effect of minocycline is similar to the PI3K inhibitor Wortmannin.

3.5. Doxycycline and minocycline increases TIMP-1 expression in VEGF-treated HASMC

TIMPs may partially regulate MMP-2, -9 enzymatic activity (Forough et al., 1996). To identify the effect of doxycycline and minocycline on TIMP-1, -2 expression, we examined their expression in conditional media from doxycycline or minocycline-treated HASMCs. We found that minocycline did not induce TIMP expression (data not shown). Interestingly, TIMP-1 expression was significantly increased in the doxycycline treated HASMCs (Figure 7). TIMP-2 expression was not detected in the conditional media from the control and doxycycline/minocycline-treated HASMCs (data not shown here).

4. Discussion

Our *in vitro* studies demonstrate that: 1) doxycycline and minocycline inhibit VEGF-induced HASMC migration; 2) both doxycycline and minocycline inhibit MMP-9 activity in a dose-dependent manner in VEGF-treated HASMCs; 3) one of the pathways of minocycline is through down-regulating PI3K/Akt signaling; and 4) the effect of doxycycline may be through up-regulating TIMP-1 expression in inhibition of VEGF-induced HASMC migration.

VEGF plays an important role in regulating endothelial cell and SMC proliferation and migration, and can be synthesized by many cell types including SMCs (Mohle et al., 1997). Our previous study demonstrated that VEGF induced human brain SMC migration (Yang et

al., 2004). However, what is unclear is which down stream molecules and signal pathways are involved in SMC migration.

VEGF and MMP are closely related and influence each other during angiogenesis (Bergers et al., 2000; Hashimoto et al., 2002; Lamoreaux et al., 1998). We detected the relationship between gelatinase activity and SMC migration induced by VEGF, and showed that HASMCs migration was inhibited by MMPs inhibitor, such as GM6001 (Yao et al., 2004). VEGF could accelerate SMC migration through up-regulating MMP-1, -3, -9 production *in vitro* (Wang et al., 1998). Doxycycline and chemically modified tetracycline (CMT) could directly inhibit MMP-2 and MMP-9 expression in human epithelial cells and rat SMCs (Bendeck et al., 2002; Nip et al., 1993; Uitto et al., 1994). These studies are consistent with the hypothesis that overexpression of VEGF in SMCs is a consequence of increased migration due to increased MMP activity.

Our results identify that doxycycline and minocycline inhibit VEGF-induced MMP-9 but not MMP-2 activity, which is parallel to our *in vivo* brain angiogenesis model (Lee et al., 2004). Although MMP-2 and MMP-9 have similar substrate specificities (Okada et al., 1992; Senior et al., 1991), there are differences in the regulation of their expression. The basal levels of MMP-9 in the brain are usually low; cytokines or growth factors can up-regulate its expression (Fabunmi et al., 1996; Galis et al., 1994). MMP-9 is an inducible protein, which responds to the stimulation of VEGF. MMP-2, as a constitutive protein, may be less responsive to growth factors such as VEGF stimuli (Fabunmi et al., 1996; Galis et al., 1994).

HASMC migration is partially regulated by endogenous MMP inhibitors, which exist in the SMCs themselves. For example, TIMP could bind and inactivate MMPs and consequently reduce SMC migration (Shapiro et al., 1992). TIMP can be released by many cell types and is also present in the serum (De Clerck et al., 1989; Stricklin et al., 1983; Welgus et al., 1983). TIMP-1 and TIMP-2 have been found in the SMCs (Roeb et al., 1993). In our study, TIMP-1 was increased especially in the doxycycline-treated HASMCs, suggesting that increased endogenous TIMP-1 could inhibit MMP-9 activity and consequently inhibit HASMC migration.

The mechanisms of the action by which doxycycline and minocycline inhibit VEGF-induced SMC migration are not completely understood. One potential explanation is that doxycycline and minocycline could bind to Zn^{++} or Ca^{++} associated with MMPs, blocking the active site or inducing conformational changes that render the proenzyme susceptible to fragmentation during activation (Golub et al., 1998; Smith et al., 1996). We found that minocycline (but not doxycycline) inhibited PI3K/Akt pathway. This effect is almost the same as Wortmannin, a specific inhibitor for PI3K/Akt signal pathway. PI3K is indispensable to cell migration induced by growth factors such as VEGF. For example, VEGF binding its receptors could lead to PI3K and down stream signaling activation (Gerber et al., 1998; Guo et al., 1995). PI3K catalyzes inositol phospholipids at the D3 position to generate PI3, 4, 5-trisphosphate and PI3, 4-bisphosphate. The 3-phosphoinositides act as potent signaling molecules to regulate cell action response to angiogenesis (Imai et al., 1999; Knall et al., 1997). Although both doxycycline and minocycline have similar anti-angiogenic effects on the inhibiting HASMC migration and MMP activity, minocycline seems more efficient because it inhibits both ERK1/2 and Akt pathways. The effect of doxycycline on the inhibiting HASMC migration could be through up-regulating TIMP-1.

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Abbreviations

ECM

extracellular matrix

HASMCs

	human aortic smooth muscle cells
MMP	matrix metalloproteinase
PI3K	phosphatidylinositol 3-kinase
TIMP	tissue inhibitors of metalloproteinase
VEGF	vascular endothelial growth factor

Figure 1. Doxycycline and minocycline inhibit HASMCs migration

Bar graph shows the effects of doxycycline and minocycline on VEGF-induced HASMC migration. Confluent HASMCs were arrested in 0.2% FBS-free medium. After resuspending HASMCs in serum-free medium, HASMCs were pre-incubated with either minocycline (7.5 to 60 μM) or doxycycline (5 to 40 μM) for 30 minutes. Then, VEGF (20 μg/ml) was added. The inhibiting effect of doxycycline and minocycline on VEGF-induced HASMC migration was determined in these experiments. Data are from three independent experiments with duplicates and are expressed as mean±SD. *, P<0.05, †, p<0.01, doxycycline or minocycline plus VEGF treated groups vs. the control (VEGF-treated) group.

Figure 2. MTT test

Bar graph shows the viability of HASMCs incubated with indicated concentration of doxycycline and minocycline. HASMCs were incubated with doxycycline and minocycline (0 or 120 μM) and VEGF, and the plates were incubated for another 36 hours; 2mg/ml MTT solution was added and the plates were incubated again for 3 hours. The final solution was measured at 570 nm using a plate reader. Data are shown as mean $\pm$ SD; n=3. *, $p<0.05$, doxycycline or minocycline treated groups vs. the control (non-treated) group.

Figure 3. Doxycycline and minocycline inhibit MMP-2 and MMP-9 activities

Effects of doxycycline and minocycline on VEGF-induced MMP activities in HASMCs were determined by MMP zymographic assay. After pretreatment of serum-free HASMCs for 24 hours, HASMCs were treated with VEGF (20 ng/ml) and doxycycline or minocycline for 24 hours. **Upper panel:** zymographic image represents one experiment of doxycycline and minocycline treatment on MMP-2 and MMP-9 expression. Standard=MMP-2/-9 zymographic standards. Lane 1 is the control (without VEGF), lane 2 is VEGF treatment (stimulate alone), and lane 3 is PD98059 treatment (positive control). Lanes 4 to 7 indicate the effects of doxycycline and minocycline on MMP activities in the VEGF treated HASMCs. **Lower panel:** Bar graphs show the quantitative zymograms. MMP levels were separately analyzed by latent and active MMP-2, and latent and active MMP-9. Data show five independent experiments with duplicates, and are expressed as mean+SD. *, indicates $p < 0.05$, doxycycline or minocycline plus VEGF treated groups vs. control (VEGF-treated) group.

Figure 4. Doxycycline and minocycline inhibit MMP-2 mRNA

Bar graph shows the effects of doxycycline and minocycline on VEGF-induced MMP-2 mRNA expression in HCSMCs using real time PCR. Data show three independent experiments with duplicates, and are expressed as mean+SD. No statistical significance was detected among groups.

Figure 5. Doxycycline and minocycline inhibit MMP-9 mRNA

Bar graph shows the effects of doxycycline and minocycline on VEGF-induced MMP-9 mRNA expression in HCSMCs using real time PCR. Cont= control group, Mino=minocycline, and Doxy= doxycycline. Data show three independent experiments with duplicates, and are expressed as mean+SD. *p<0.05, and †p<0.01, doxycycline or minocycline plus VEGF treated groups vs. control (VEGF-treated) group.

Figure 6. Minocycline inhibits PI3K/Akt pathway

The effects of doxycycline and minocycline on VEGF-induced PI3k/Akt expression in HASMCs were determined by Western blot analysis. After pretreatment of serum-free HASMCs for 24 hours, HASMCs were co-treated with doxycycline/VEGF (20 ng/ml) or minocycline/VEGF (20 ng/ml) for 24 hours. Bar graphs show Western blot analysis results. Minocycline but not doxycycline inhibited VEGF-induced Akt/PKB phosphorylation in the HASMCs. Cont= control group, Wort=wortmannin, Mino=minocycline, and Doxy=doxycycline. Data are shown as mean±SD; n=5. * =p<0.05 and †=p<0.01, doxycycline or minocycline plus VEGF-treated groups vs. control (VEGF-treated) group.

Figure 7. Doxycycline up-regulates TIMP-1 expression

The effects of doxycycline on VEGF-induced TIMP-1 protein in HASMCs were determined by Western blot analysis. Upper panel represents an experiment of TIMP-1 detection. Lane 1 is the control (without VEGF) and lane 2 is positive control. Lanes 3 and 4 indicate the treatment with doxycycline in the HASMCs. Bar graphs show Western blot analysis results. Doxycycline up-regulated TIMP-1 in the HASMCs. Cont= control group, D15= doxycycline 15 μ M, and D30= doxycycline 30 μ M. Data are shown as mean $\pm$ SD; n=5, * = $p < 0.05$ and †= $p < 0.01$, doxycycline plus VEGF treated groups vs. control (VEGF-treated) group.