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Scientific Research

# Inhibitory Actions of Tetrandrine on Tumor Necrosis Factor α-Induced NF-κB Activation in Neovascularization of Cultured Choroidal Explants*

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## Abstract

Tetrandrine (1 μM), a *bis*-benzylisoquinoline alkaloid isolated from *Stephania tetrandra* S Moore, significantly decreased tumor necrosis factor alpha (TNFα ; 10 ng/ml)-induced increase in the number of micro vessels that budded from cultured rat choroidal explants. Tetrandrine also decreased the TNFα-induced increase in the number of cells composing the microvessels. Ammonium pyrrolidine dithiocarbamate (APDC; 0.1-0.3 μM), an inhibitor of nuclear factor-κB (NF-κB), decreased the TNFα-induced increase in the number of microvessels in a concentration-dependent manner. TNFα increased the phosphorylation and degradation of inhibitor of NF-κB (IκBα), as well as increasing the DNA-binding activity of NF-κB in choroidal explants. TNFα induced an increase of vascular endothelial growth factor (VEGF)-A mRNA, but not VEGF-C mRNA or VEGF-D mRNA. TNFα-induced angiogenic action was inhibited by treatment of VEGF-A antibody in cultured choroidal capillaries. Tetrandrine inhibited the TNFα-induced increases of phosphorylation and degradation of IκBα, and reduced the TNFα-induced increase of DNA-binding activity of NF-κB in choroidal explants. In conclusion, tetrandrine inhibits TNFα-induced activation of NF-κB in the choroidal capillaries via inhibition of TNFα-induced phosphorylation of IκBα.

**Keywords:** Choroidal Neovascularization, Anti-angiogenesis, Tetrandrine, Tumor Necrosis Factor α, NF-κB Activity, Phosphorylation of IκBα

## 1. Introduction

The *bis*-benzylisoquinoline alkaloid tetrandrine is a component of *Stephania tetrandra* S. Moore, which is a herb used in traditional Chinese medicine to treat patients with silicosis, asthma, hypertension and arthritis [1-4]. This alkaloid has been shown to be effective in experimental models of allergic encephalitis, airway microvascular leakage, subcutaneous air pouch inflammation, granuloma angiogenesis in chronic inflammation and proliferation of synovial cells [5-8]. Tetrandrine inhibits the production and release of a broad range of in lammatory mediators and cytokines [9]. It has been reported to block voltage-dependent L-type and T-type $Ca^{2+}$ channels in various cells, including retinal cells [10], and to inhibit nitric oxide (NO) production in endothelial cells by blocking the $Ca^{2+}$ release-activated $Ca^{2+}$ channel [11]. Tetrandrine also inhibits hypoxia-induced neovascularization of the small pulmonary artery [12], protects islet beta cells from alloxan-induced injury [13], and prevents spontaneous development of diabetes mellitus in BB rats [14]. Moreover, it has been proposed that $Ca^{2+}$ flux and NO production in endothelial cells are associ-

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ated with angiogenesis induced by vascular endothelial growth factor (VEGF) [15-16]. Administration of tetrandrine suppresses neovascularization of the choroidal capillaries, which is facilitated by VEGF and platelet-derived growth factor (PDGF)-B in culture [17]. Choroidal tissue in streptozotocin (STZ)-induced diabetic rats accumulates $N^{\varepsilon}$-(carboxymethyl) lysine (CML), one of the advanced glycation end products (AGEs) (unpublished data). CML facilitates neovascularization of choroidal capillaries in culture [18-21]. Tetrandrine inhibits diabetic state-induced overproduction of choroidal and retinal neovascularization in culture [17,22,23].

Tumor necrosis factor (TNF) α is proinflammatory cytokine that has been implicated in the pathogenesis of diabetic retinopathy [24,25]. It is found in the extracellular matrix, endothelium and vessel walls of fibrovascular tissue in the eyes of patients with proliferative diabetic retinopathy [25] and vitreous humor from eyes of patients with diabetic complications [26]. This cytokine has been utilized as a potent promoting factor in *in vivo* and *in vitro* angiogenesis models [27-29]. TNFα can induce the expression of many important immune-and angiogenesis-related genes through TNFα receptors. TNFα signaling is required for vessel development in ischemia-induced neovascularization [30]. The increased expression of many inflammatory proteins is mediated at the level of gene transcription through the activation of proinflammatory transcription factors, including nuclear factor kappa B (NF-κB) [31].

In the present study, we investigated the effect of tetrandrine on TNFα-induced cell signaling pathways in neovascularization of choroidal capillaries.

## 2. Materials and Methods

### 2.1. Materials

The chemical structure of tetrandrine is shown in **Figure 1.** Tetrandrine, fibrinogen and thrombin were from Sigma (St. Louis, MO, USA); ε-aminocaproic acid, dimethyl sulfoxide, collagenase and ISOGEN® were from Wako Pure Chemical Industries Ltd. (Osaka, Japan), dNTPs, RNase inhibitor, M-MLV reverse transcriptase, fetal bovine serum (FBS), oligo $dT_{12\text{-}18}$ primer and Taq DNA polymerase were from Invitrogen (Carlsbad, CA, USA); anti-IκBα antibody and anti-phospho-IκBα antibody were from Cell Signaling Technology, Inc. (Beverly, MA, USA); TNFα and anti-VEGF were from R&D Systems (Minneapolis, MN, USA); ECL Plus western blotting detection system was from GE Health Biosciences (Piscataway, NJ, USA); probes for NF-κB was from Promega K. K. (Tokyo, Japan); Dulbecco's modified Eagle's medium (DMEM) was from Nissui (Tokyo); penicillin/streptomycin was from Lonza (Walkersville, MD, USA); qPCR Mastermix for SYBR® Green I was from Eurogentec (San Diego, CA, USA); ammonium pyrrolidine dithiocarbamate (APDC) was purchased from Dojin (Osaka).

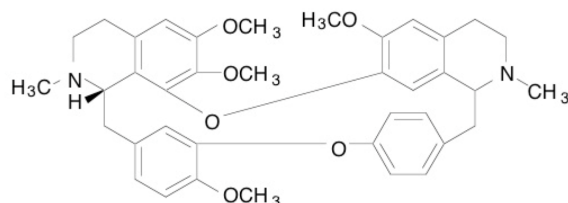


**Figure 1. Chemical structure of tetrandrine.**

### 2.2. Culture of Choroidal Tissues

All of the animal experiments were performed in accordance with the guidelines of the Committee on Animal Experiments in Hokuriku University. Male Wistar rats (7-9 weeks of age) were purchased from Nippon SLC (Shizuoka, Japan). The rats were anaesthetized and killed, and eyeballs were rapidly isolated under aseptic conditions. Choroidal explants were isolated under an optical microscope as previously reported [18-20,32]. The size of isolated explants was approximately 0.16 $mm^2$. The explants were plated on fibrin gels prepared by mixing 3 mg fibrinogen (0.3 ml), and 1 U thrombin per ml DMEM containing 30 U/ml penicillin/30 μg/ml streptomycin in 24-well plates. Equal volumes of a mixture of the above concentrations of fibrinogen and thrombin were overlaid and allowed to solidify. The explants were cultured with 1% FBS-DMEM (0.5 ml) containing 30 U/ml penicillin/ 30 μg/ml streptomycin and 300 μg/ml ε-aminocaproic acid in a $CO_2$ incubator at 37°C for 8 days. In some experiments, tetrandrine (0.1 and 1 μM), APDC (0.1 and 0.3 μM), anti-VEGF antibody (0.3 μg/ml) or TNFα (10 ng/ml) were added to tissue culture.

### 2.3. Assessment of Neovascularization

The number of micro vessel-like structures newly budded from choroidal explants cultured as described in the previous section was counted under a CKS microscope equipped with a camera (Olympus, Tokyo). The microvessel-like structures have been demonstrated previously to have the features of immature microvessels, consisting of vascular endothelial progenitor cells, such as angioblasts [18]. The number of microvessel-like structures per explant was used as an index of *in vitro* neovascularization.

Choroidal tissue from eyeballs of male Wistar rats (7-9 weeks of age) was sampled with a biopsy punch (1

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mm; Kai Industries Co. Ltd., Gifu, Japan). The choroidal explants (0.785 $mm^2$) were cultured in fibrin gel with 1% FBS-DMEM containing antibiotics and ε-aminocaproic acid in a $CO_2$ incubator at 37°C for 8 days as described in the previous section. The primary choroidal explants were removed, and microvessel-like structures were digested with 0.75% collagenase (1 ml) in phosphate-buffered saline (PBS) in a $CO_2$ incubator at 37°C for 30 min. The digested cells were transferred to 1.5 ml centrifuge tubes, and centrifuged at 16,000 xg for 5 min. Total cell number was counted using a Burker-Turk blood cell counting plate (Erma Inc., Tokyo) under a CKZ microscope (Olympus).

## 2.4. DNA Binding Activity of NF-κB

Choroidal tissues isolated from eyeballs of male Wistar rats (7-9 weeks of age) were cut into pieces of approximately 0.16 $mm^2$, and incubated in 1% FBS-DMEM containing TNFα (10 ng/ml) with or without tetrandrine (1 μM) for 120 min in a $CO_2$ incubator at 37°C. The choroidal tissues were lysed with 50 μl of buffer A (10 mM HEPES; pH 7.9, 10 mM KCl, 0.1% Triton X-100, 50 μM DTT, 1.5 mM $MgCl_2$) for 15 min on ice. After centrifugation at 250 xg for 5 min at 4°C, 20 μl of buffer A was added to the pellets, and the mixture was incubated for 5 min on ice and centrifuged again at 8,000 xg for 20 min at 4°C. The pellets were incubated in buffer B (20 mM HEPES; pH 7.9, 420 mM NaCl, 1% CA-630, 25% glycerol, 500 μM DTT, 1.5 mM $MgCl_2$, 0.2 mM EDTA) for 60 min on ice. After centrifugation at 16,000 xg for 5 min at 4°C, the supernatants were harvested as nuclear protein extracts and adjusted to 1 mg/ml proteins. The DNA binding reaction was performed with 3 μg of nuclear protein and 15,000 cpm of a $^{32}P$-labeled oligonucleotide containing NF-κB-binding element (5'-AGT TGA GGG GAC TTT CCC AGC C-3'; Promega) as described previously [33,34]. The samples were separated on 5% polyacrylamide gels, which were analyzed with a Typhoon 9410 image analyzer (Amersham Biosciences).

## 2.5. Western Blotting

Choroidal tissues isolated from eyeballs of male Wistar rats (7-9 weeks of age) were cut into pieces of approximately 0.16 $mm^2$, and incubated in 1% FBS-DMEM containing TNFα (10 ng/ml) with or without tetrandrine (1 μM) for 5 min in a $CO_2$ incubator at 37°C. The choroidal tissues were extracted with 200 μl of triple-deter-gent buffer (50 mM Tris-HCl; pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1% Nonidet P-40, 0.5% sodium deoxycholate for 30 min on ice, and homogenized using an UltraS Homogenizer VP-5S (Taitec, Saitama, Japan). After centrifugation at 16,000 xg for 5 min at 4°C, the supernatants were harvested as samples for Western blotting; results were normalized with respect to untreated controls. Western blotting analysis was performed using antibodies against IκBα and its phosphorylated proteins as described previously [33,34]. Antibody-bound proteins were detected using an ECL-Plus Western Blotting Kit (GE Health Biosciences) and analyzed with a Typhoon 9410 image analyzer.

## 2.6. Real-Time PCR

Total RNAs from the cultured choroidal explants were extracted using ISOGEN® according to the manufacturer's instructions. Then, reverse transcription reaction was performed for 1 h at 37°C in a final volume of 20 μl, containing 3 μg of total RNA (previously denatured for 10 min at 65°C), 0.5 mM dNTPs, 25 ng/ml oligo $dT_{12-18}$ primer and 200 units of M-MLV reverse transcriptase. Real-time PCR was optimized with each set of oligonucleotide primers, which were designed for rat VEGF-A (Gene Bank accession No. NM_031836; forward, 5'-GCA CTG GAC CCT GGC TTT ACT-3'; reverse, 5'-CGC TGG TAG ACG TCC ATG AA-3'; amplicon size 119 bp), rat VEGF-C (Gene Bank accession No. NM_053653; forward, 5'-CAC AAT CAG TTT TGC CAA TCA CA-3'; reverse, 5'-CAC ACG TAG TTT GCT GGA CAA GTC-3'; amplicon size 127 bp), rat VEGF-D (Gene Bank accession No. AY032728; forward, 5'-CAC CGA GCA GTG AAG GAT GTT-3'; reverse, 5'-CAG TCC TCA GAG TGT GCG ACT T-3'; amplicon size 124 bp) and rat β-actin (Gene Bank accession No. NM_031144; forward, 5'-AGG GAA ATC GTG CGT GAC AT-3'; reverse, 5'-GAA CCG CTC ATT GCC GAT AG-3'; amplicon size 149 bp). Primers were designed with the help of the software Primer Express® Ver. 3.0 (Applied Biosystems Japan, Tokyo). The reactions were performed using qPCR Mastermix for SYBR® Green I in a 25 μl reaction volume containing 10 μl of cDNA, 10 pmol of each primer and 12.5 μl of the 2x Mastermix in an ABI PRISM® 7500 (Applied Biosystems Japan). The program profile used for amplification was: 50°C for 2 min (stage 1), 95°C for 10 min (stage 2), 40 cycles of denaturation at 95°C for 15 sec and annealing at 65°C for 1 min (stage 3), 95°C for 15 sec (stage 4), 60°C for 1 min (stage 5), and 95°C for 15 sec (stage 6). The real-time amplified PCR products were checked on polyacrylamide gel to demonstrate that, under the specified real-time conditions, the expected amplicon sizes are produced.

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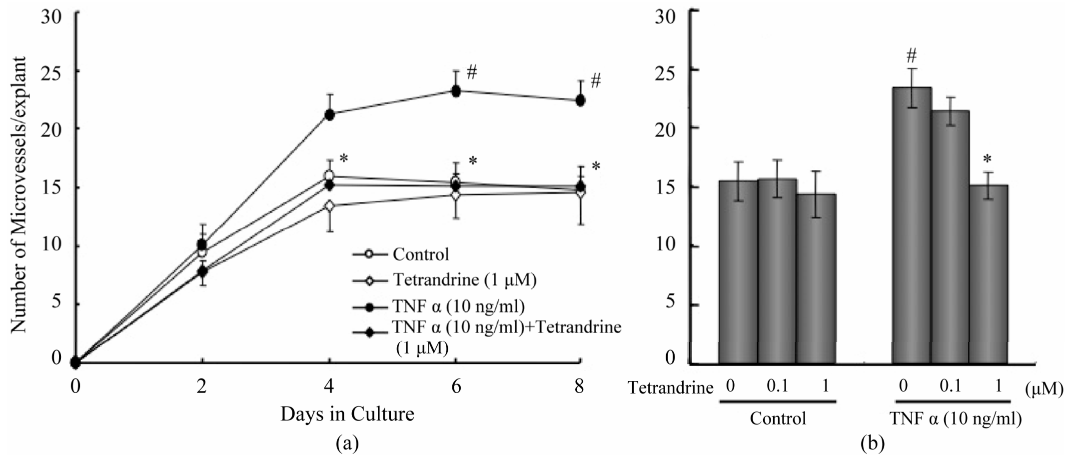


**Figure 2. Effect of tetrandrine on the increase in the number of microvessels induced by TNFα in rat choroidal explants. a, The choroidal explants were cultured in the presence or absence of TNFα (10 ng/ml) and/or tetrandrine (1 μM) for 8 days. b, The explants were incubated with tetrandrine (0, 0.1 and 1 μM) in the presence (right) or absence (left) of TNFα (10 ng/ml) on the 6th day in culture. Values are expressed as means ± S.E.M. of at least 3 data. #P < 0.05 vs. the corresponding control without TNFα nor tetrandrine. *P < 0.05 vs. TNFα alone without tetrandrine.**

## 2.7. Statistical Analysis

Values were expressed as means ± S.E.M. of at least 3 data. Statistical analysis was carried out using Student's *t*-test and Welch's *t*-test at P = 0.05 or P = 0.01. A value of P < 0.05 was considered statistically significant.

# 3. Results

## 3.1. Inhibitory Effects of Tetrandrine on TNFα-Induced Increase of Choroidal Neovascularization and Cell Proliferation in Choroidal Microvessels in Culture

We have previously reported that TNFα increased neovascularization of cultured choroidal explants in normal Wistar rat (Kobayashi *et al.*, 2005). Tetrandrine (1 μM) in **Figure 1** suppressed 10 ng/ml TNFα-induced increase in the number of microvessels that budded from choroidal capillaries from the 4th day in culture, and this inhibitory action was in a concentration-dependent manner (**Figure 2**). TNFα (10 ng/ml) increased the number of cells in microvessels of choroidal explants at the 8th day in culture (**Figure 3**). Tetrandrine (1 μM) completely reduced the TNFα-induced increase in the number of cells in microvessels budded on the 8th day in culture to the levels of control with tetrandrine (**Figure 3**). However, tetrandrine (1 μM) did not significantly affect the number of microvessels (**Figure 2**) and cell number of microvessels (**Figure 3**) in the absence of TNFα, respectively.

## 3.2. Inhibitory Effect of APDC on TNFα-Induced Increase of Choroidal Neovascularization

To study the participation of NF-κB activity in signal transduction of TNFα, the effect of APDC, an inhibitor of NF-κB, on TNFα-induced choroidal neovascularization in culture was investigated. APDC (0.1-0.3 μM) significantly reduced the TNFα (10 ng/ml)-induced increase in the number of microvessels that budded from choroidal capillaries from the 4th day in culture, and the inhibitory action was in a concentration-dependent manner. However, APDC did not affect the number of microvessels in the absence of TNFα (**Figure 4**). This

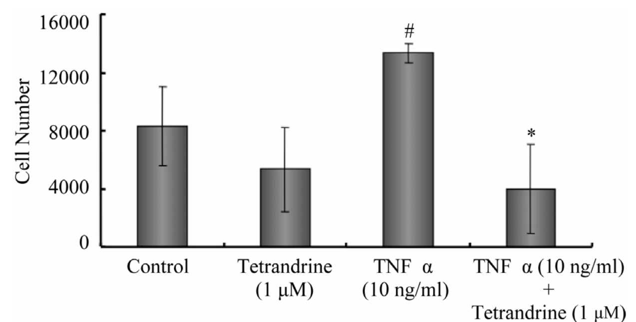


**Figure 3. Effect of tetrandrine on the increase of the cell number in microvessels induced by TNFα in rat choroidal explants. Microvessels that budded from the explants in the presence or absence of TNFα (10 ng/ml) and/or tetrandrine (1 μM) for 8 days in culture were digested with collagenase for 30 min. The cell number in the microvessels per explant was counted. Values are expressed as means ± S.E.M. of at least 3 data. #P < 0.05 vs. control without agents, *P < 0.05 vs. TNFα alone without tetrandrine.**

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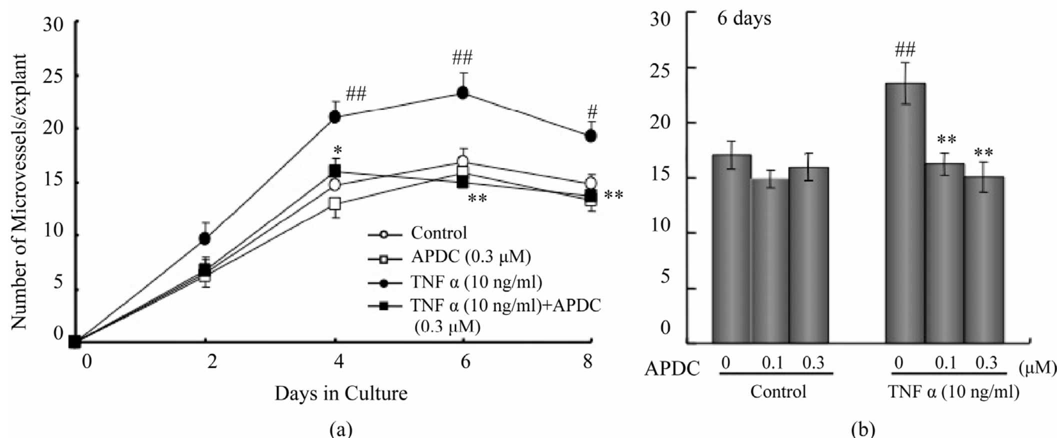


**Figure 4. Effect of APDC on the increase in the number of microvessels induced by TNFα in rat choroidal explants. a, The choroidal explants were cultured in the presence or absence of TNFα (10 ng/ml) and/or APDC (0.3 μM) for 8 days. b, The explants were incubated with APDC (0, 0.1 and 0.3 μM) in the presence (right) or absence (left) of TNFα (10 ng/ml) on the 6th day in culture. Values are expressed as means ± S.E.M. of at least 3 data. # P < 0.05, ##P < 0.01 vs. the corresponding control without TNFα or APDC. *P < 0.05, **P < 0.01 vs. TNFα alone without APDC.**

effect of APDC showed a similar pattern to that of tetrandrine.

### 3.3. Inhibitory Effect of Tetrandrine on the TNFα-Induced Increase of DNA Binding Activity of NF-κB

The effect of tetrandrine on the TNFα-induced increase of DNA binding activity of NF-κB was investigated using the nuclear fraction of choroidal explant incubated for 120 min. Tetrandrine (1 μM) reduced the TNFα (10 ng/ml) -induced increase of DNA binding activity, though not to the level seen in the absence of TNFα (**Figure 5**). The NF-κB specific bands were confirmed by the competition experiments with unlabeled NF-κB binding oligonucleotides (related competitor) and unlabeled AP-1 binding oligonucleotides (unrelated competitor) (data not shown).

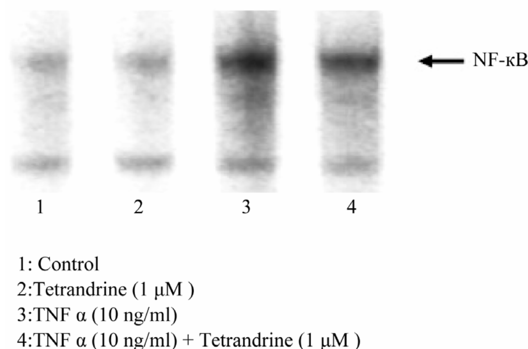


**Figure 5. Effect of tetrandrine on the TNFα-induced increase of DNA binding activity of NF-κB. The choroidal explants were preincubated with or without tetrandrine (1 μM) for 30 min and then TNFα (10 ng/ml) was added for 120 min. DNA binding activity of NF-κB was determined by means of gel shift assay.**

### 3.4. Effect of Tetrandrine on the TNFα-Induced Increases of Phosphorylation and Degradation of IkBα

To study the mechanism of tetrandrine's action on NF- κB activation by TNFα, the effect of tetrandrine on phosphorylation of IκBα and on the content of IκBα in choroidal capillary cells treated with TNFα was investigated. TNFα (10 ng/ml) increased the content of phosphorylated IκBα and decreased the content of total IκBα in the choroidal explants. Tetrandrine (1 μM) inhibited both actions of TNFα on phosphorylated IκBα and total IκBα (**Figure 6**).

### 3.5. Effect of Anti-VEGF Antibody on TNFα-Induced Choroidal Neovascularization

To examine whether production of VEGF is involved in

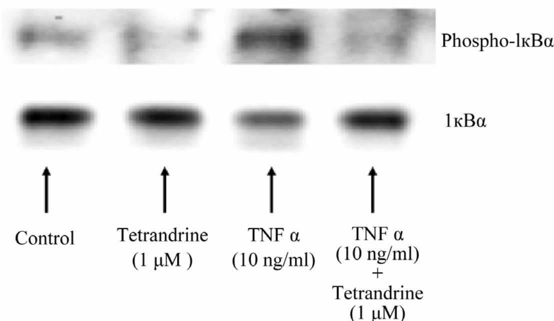


**Figure 6. Effect of tetrandrine on the TNFα-induced increase of phosphorylation and degradation of IkBα in choroidal explants. The choroidal explants were preincubated with or without tetrandrine (1 μM) for 30 min and then TNFα (10 ng/ml) was added for 30 min. The contents of phosphorylated IκBα and total IκBα were determined by Western blotting.**

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the TNFα-induced increase of choroidal neovascularization, the effect of anti-VEGF antibody on the neovascularization was investigated in culture. Simultaneous treatment of anti-VEGF antibody (0.3 μg/ml) significantly reduced the TNFα (10 ng/ml) - induced increase of choroidal neovascularization on the $6^{th}$ and $8^{th}$ days in culture (**Figure 7**).

### 3.6. Expression of VEGF mRNA during TNFα- Induced Choroidal Neovascularization

The levels of mRNAs of VEGF-A, VEGF-C and VEGF-D in choroidal explants were directly measured on the $6^{th}$ day in culture by means of real-time PCR and normalized by β-actin mRNA levels. Expression of normalized VEGF-A mRNA in choroidal explants was almost 5-time greater than those of VEGF-C and VEGF-D. TNFα (10 ng/ml) significantly increased expression of VEGF-A mRNA 3-time greater. However, TNFα unaffected VEGF-C mRNA expression and decreased VEGF-D mRNA expression in choroidal explants, respectively (**Table 1**).

## 4. Discussion

The present study was designed to investigate the action of tetrandrine on TNFα-induced cell signaling associated with neovascularization of choroidal capillaries in culture. Tetrandrine inhibited 1) the TNFα-induced increase of choroidal neovascularization (**Figures 2** and **3**), 2) the TNFα-induced increase of DNA binding activity of

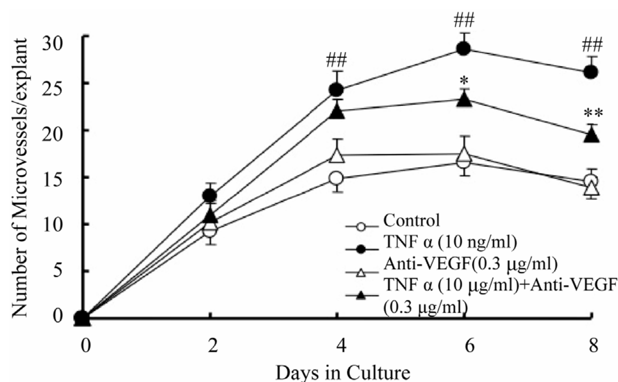


**Figure 7. Effect of anti-VEGF antibody on the TNFα-induced increase in the number of microvessels in choroidal explants in culture. The choroidal explants were cultured in the presence or absence of TNFα (10 ng/ml) and/or anti-VEGF antibody (0.3 μg/ml) for 8 days. Values are expressed as means ± S.E.M. of at least 3 data. ##P < 0.01 vs. the corresponding control without TNFα or tetrandrine. *P < 0.05, **P < 0.01 vs. TNFα alone without anti-VEGF antibody at the corresponding day.**

**Table 1. mRNA Levels of VEGF-A, VEGF-C and VEGF-D in choroidal explants cultured in the presence of TNFα.**

| | VEGF-A | VEGF-C | VEGF-D |
|---|---|---|---|
| TNF | 0.1664 ± 0.0237* | 0.0018 ± 0.0010 | 0.0008 ± 0.0005* |
| Control | 0.0531 ± 0.0113 | 0.0023 ± 0.0005 | 0.0025 ± 0.0014 |

The choroidal explants were cultured in fibrin gel with 1% FBS-DMEM with or without TNFα (10 ng/ml) for 6 days. VEGF-A, VEGF-C and VEGF-D mRNA levels (△C) in choroidal explants were determined by means of the protocol for real-time reverse transcription- polymerase chain reaction and normalized by β-actin mRNA levels. Values were expressed as means ± S.E.M. of at least 3 data. *$P$ < 0.05 vs. the corresponding control without TNFα.

NF-κB (**Figure 5**), 3) the TNFα-induced increase of phosphorylation of IκBα (**Figure 6**) and 4) the TNFα-induced increase of degradation of IκBα in choroidal explants (**Figure 6**). These results indicate that tetrandrine inhibits TNFα-induced neovascularization through NF-κB inactivation in choroid. The site of action of tetrandrine is upstream of TNFα-induced phosphorylation in the process of NF-κB activation. Therefore, tetrandrine decreases translocation of NF-κB heterodimers into the nucleus of choroidal capillary cells. This inhibitory action of tetrandrine is similar to that of APDC in choroidal neovascularization. APDC prevented release of IκB from NF-κB in intact cells, but did not inhibit the DNA binding activity in the nucleus *in vitro* or the inducibility of AP-1 by PMA. APDC has at least two chemical properties that may be related to the inhibition of NF-κB activity: one is a chelating activity for heavy metals, and the second is an antioxidant activity [35].

We have previously reported that nifedipine, an L-type $Ca^{2+}$ channel blocker, inhibits TNFα-induced neovascularization of choroidal capillaries [32]. Nifedipine also inhibits basic fibroblast growth factor-induced proliferation of endothelial cells by causing cell-cycle arrest at $G_0/G_1$ through blocking the $Ca^{2+}$ channel [36]. There are also evidences that nifedipine results in increased release of NO [37-38]. It has been proposed that $Ca^{2+}$ flux and NO production in endothelial cells are associated with angiogenesis induced by VEGF [15-16]. Tetrandrine also blocks voltage-dependent L-type and T-type $Ca^{2+}$ channels in various cells, including retinal cells [10], and inhibits NO production in endothelial cells by blocking $Ca^{2+}$ release-activated $Ca^{2+}$ channels [11]. The action of tetrandrine on TNFα-induced cell signaling in neovascularization may therefore be associated with the inhibition of NF-κB activity through reducing $Ca^{2+}$ flux and NO production in choroidal capillaries. Angiogenic action of TNFα has been reported to be associated with

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increased expression of many immune-and angiogenesis-related genes, mediated by TNFα receptors. TNFα signaling induces NF-κB activation and regulates VEGF expression and consequently VEGF- mediated neovascularization [30,39,40]. On the contrary NF-κB also induces formation of vascular endothelial growth inhibitor (VEGI), which induces apoptosis to proliferating cells while enforcing a growth arrest in quiescent cells [41]. In the present study, TNFα accelerated cell proliferation in newly formed microvessels, as well as number of microvessels budded from cultured choroidal capillaries (**Figures 2** and **3**), indicating that TNFα may induce expression of VEGF rather than expression of VEGI in our angiogenesis model of choroidal explants. We have additional data that TNFα (10 ng/ml) inhibits cleavage of poly (ADP-ribose) polymerase by caspase-3 (data not shown), supporting that TNFα is angiogenic activator but not inducer of apoptosis in the choroidal explants. The angiogenic action of TNFα on choroidal neovascularization was inhibited by APDC, an inhibitor of NF-κB [35] (**Figure 4**), supporting that NF-κB is involved in the mechanism of TNFα's action on choroidal neovascularization. The present investigations also indicated that TNFα facilitated phosphorylation of IκBα and degradation (decrease of content) of IκBα (**Figure 6**). The degradation of IκBα results from release of NF-κB heterodimers to translocate to the nucleus, where they bind to nuclear DNA (**Figure 5**), leading to activation of specific subsets of angiogenic genes in the choroidal explants. We have now plans to identify cellular sources of NF-κB and I-κB in our choroidal explant lysates.

The present study directly demonstrates that TNFα increases expression of mRNA of VEGF-A, but not VEGF-C or VEGF-D in choroid (**Table 1**). Angiogenic action of TNFα was reduced by anti-VEGF-A antibody in choroidal explant (**Figure 7**). These results indicate that TNFα stimulates the production and release of VEGF-A via activation of NF-κB in choroidal explant and increases neovascularization. VEGF-C and VEGF-D interact with VEGF receptor (VEGFR)-2 and VEGFR-3 and have a role for the growth of lymphatic vessels [42]. TNFα did not increase mRNA of VEGF-C and VEGF-D, suggesting that TNFα does not increase lymph angiogenesis. Tetrandrine (1 μM) inhibited TNFα-induced cell signaling of neovascularization in cultured choroidal explant. However, this concentration of tetrandrine did not affect basal neovascularization or cell proliferation in the absence of TNFα (**Figures 2** and **3**). Tetrandrine (1 μM) inhibits VEGF-induced neovascularization in choroidal explant [17], indicating that tetrandrine reduces both of TNFα-induced release of VEGF-A and the action of VEGF-A in neovascularization of choroidal explant.

## 5. Conclusion

Tetrandrine inhibits TNFα-induced activation of NF-κB in the choroidal capillaries via inhibition of TNFα-induced phosphorylation of IκBα in choroidal neovascularization.

## 6. Acknowledgements

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