Purpose. To investigate the effects of 99mTc-MDP, a decay product of 99mTc-MDP, on the development of choroidal neovascularization (CNV), together with its underlying mechanisms.

Methods. C57BL/6 mice were used to induce CNV by laser photocoagulation. 99mTc-MDP at the doses of 0.5 × 10⁻¹³, 1 × 10⁻¹⁴, and 2 × 10⁻¹⁴ μg/kg or the same volume of PBS was intraperitoneally injected daily after photocoagulation until the end of the experiment. Seven days after laser injury, mice were perfused with fluorescein-labeled dextran, and areas of CNV were measured. Numbers of infiltrating macrophages, protein levels of VEGF, and inflammation-related molecules including intercellular adhesion molecule (ICAM)-1, tumor necrosis factor (TNF)-α, and matrix metalloproteinases (MMPs) in the RPE-choroid complex were detected 3 days after laser photocoagulation. Effects of 99mTc-MDP on VEGF-induced endothelial cell migration and tube formation were also studied. Toxicity of 99mTc-MDP was evaluated in vivo and in vitro.

Results. Areas of CNV were significantly suppressed by 99mTc-MDP treatment without toxicity to the retina compared with PBS treatment in a dose-dependent manner: 99mTc-MDP treatment of 0.5 × 10⁻¹³ μg/kg (5698.60 ± 1037.70 μm²), 1 × 10⁻¹⁴ μg/kg (3678.54 ± 1328.18 μm²), and 2 × 10⁻¹⁴ μg/kg (2565.78 ± 923.80 μm²) suppressed the development of CNV by 36.12%, 58.76%, and 73.48%, respectively, compared with that in the PBS treatment group (8920.36 ± 1097.29 μm²; P < 0.001). 99mTc-MDP treatment led to significant inhibition of macrophages infiltrating to CNV together with downregulated protein expressions of VEGF, ICAM-1, TNF-α, and MMP-2. 99mTc-MDP also showed an inhibitive effect on cell proliferation and VEGF-induced migration and capillary-like tube formation of endothelial cells.


Choroidal neovascularization (CNV) is the key pathogenesis in exudative form of age-related macular degeneration (AMD), the leading cause of blindness among the elderly in developed countries. CNV is immature new blood vessel that penetrates the Bruch’s membrane and extends into the subretinal or subretinal pigment epithelium space. It often leads to loss of central vision by exudation and hemorrhage, retinal pigment epithelial (RPE) or retinal detachment, photoreceptor degeneration, and the formation of disciform scars. The exact mechanisms of CNV formation have not been fully elucidated. Recent studies demonstrate an important role of inflammatory mechanisms in CNV of AMD patients and in experimental CNV models. The inflammatory processes include macrophage infiltration and a variety of inflammatory cytokine networks. This concept of inflammation-mediated neovascularization is further supported by studies that generalized macrophage depletion reduced the size and leakage of laser-induced CNV associated with decreased macrophage infiltration and VEGF protein expression. 99mTc-MDP also showed an inhibitive effect on cell proliferation and VEGF-induced migration and capillary-like tube formation of endothelial cells.

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To our knowledge, no data have been presented concerning the effects of $^{99}$Tc-MDP on CNV development. Here we report the first evidence of the in vivo and in vitro inhibitive effects of $^{99}$Tc-MDP on the development of CNV together with the underlying cellular and molecular anti-inflammatory mechanisms.

**MATERIALS AND METHODS**

**Preparation of $^{99}$Tc-MDP**

Yunke (Chengdu Yunke Pharmaceutical, Sichuan, China) contains two preparations. Preparation A is a 5-mL vial of colorless transparent solution containing 0.05 $\mu$g $^{99}$Tc. Preparation B is a lyophilized white powder that contains 5 mg methylene diphosphonic acid (MDP) and 0.5 mg stannous chloride. $^{99}$Tc-MDP should be prepared before use by injecting preparation A into preparation B. According to the instruction manual, both preparations should be stored at 2°C to 8°C in a dark environment.

**Animals**

Male C57BL/6j mice aged 6 to 8 weeks (SPF level) were purchased from the Animal Experiment Center of Sun Yat-sen University. All animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals used in this study were treated in accordance with the ARVO Statement for the Use of Animals in Vision Research.

**Induction of CNV and Intraperitoneal Injection of $^{99}$Tc-MDP**

Mice were anesthetized by intramuscular injection of 100 mg/kg body weight (BW) ketamine hydrochloride, and pupils were dilated with 0.5% tropicamide-phenylephrine ophthalmic solution (Mydrin; Santen Pharmaceutical, Osaka, Japan). Four laser burns (50-mu spot size, 0.05-second duration, 250-mW power) were placed at a distance of 2 to 3 PD around the optic nerve with a frequency-doubled Nd:YAG laser delivery system (Haag-Streit, Mason, OH) and a coverslip as a contact membrane, which leads to the formation of CNV.20 Therefore, only burns where the laser point is thought to be the sign of disruption of Bruch's membrane, which leads to the formation of CNV.20 Therefore, only burns with bubbles were included in this study. The treatment groups were intraperitoneally injected daily until the end of experiment with $^{99}$Tc-MDP at the dose of $0.5 \times 10^{-1} \mu$g/kg BW, $1 \times 10^{-1} \mu$g/kg BW, or $2 \times 10^{-1} \mu$g/kg BW immediately after laser photocoagulation and the control group was intraperitoneally injected daily with the same volume of PBS.

**Preparation and Quantitative Analysis of Choroidal Flatmounts**

One week after laser photocoagulation, mice were anesthetized and perfused with 1 mL PBS containing 50 mg/mL fluorescein-labeled dextran (2 \times 10^6 average MW; Sigma, St. Louis, MO) through the left ventricle, as previously described.21 The eyes were enucleated and fixed in 4% paraformaldehyde for 1 hour at room temperature. The anterior segment and neurosensory retina were carefully removed; after that, four to six radial relaxing incisions were made from the edge to the equator to allow the remaining RPE-choroid-sclera complex to be flattened on a glass slide with the RPE facing up. Then a coverslip was placed on the choroidal flatmount with mounting medium (Vectashield; Vector Laboratories, Burlingame, CA). CNVs on the flatmounts were examined by fluorescence microscopy (Axioplan 2 imaging; Zeiss, Thornwood, NY), and the CNV images were digitized. Areas of CNV that were associated with hyperfluorescence (n = 13 in each group) were then measured (Image-Pro Plus 6.0 software; Media Cybernetics, Silver Spring, MD).

**Immunofluorescence for Infiltrating Macrophages**

Whole mounts of RPE-choroid-sclera complexes were obtained 3 days after photocoagulation (11–12 mice were used in each group). The complexes were incubated at 4°C overnight with a goat polyclonal antibody against mouse PECAM-1 (CD31; Santa Cruz Biotechnology, Santa Cruz, CA) and a rat polyclonal antibody against F4/80 (Serotec, Oxford, UK) followed by incubation with Avidin-Alexa 488–tagged and Avidin-Alexa 555–tagged secondary antibodies (Alexa 488 and Alexa 555; Invitrogen, Carlsbad, CA) for 2 hours at room temperature, as described previously.22 After that, PECAM-1–stained CNV areas and F4/80 positive infiltrating macrophages were scanned with a scanning laser confocal microscope (LSM710; Zeiss) in 5-mu steps, and numbers of macrophages per laser lesion were calculated by image processing and analysis software (Imaris 7.0; Bitplane, Zurich, Switzerland).

**ELISA for VEGF and Inflammation-Related Molecules**

Three days after laser photocoagulation, RPE-choroid complexes were harvested from the RPE-choroid-sclera flatmount under a dissecting microscope, using a slit knife with its edge completely dull. After that, two RPE-choroid complexes from the right and left eyes of the same mouse were placed together into 500 $\mu$L lysis buffer supplemented with protease inhibitors (Sigma), as previously described,23 and then were carefully homogenized with glass tissue homogenizer. Then the lysate was cleared of debris by centrifugation at 12,000 rpm for 10 minutes at 4°C. The levels of VEGF (n = 7 or 8 mice in each group), ICAM-1 (n = 12), and TNF-a (n = 12) in the supernatant were determined with the mouse VEGF, ICAM-1, and TNF-a ELISA kits (R&D Systems Inc., Minneapolis, MN) and normalized to total protein (determined by DC protein assay kit; Bio-Rad Laboratories) according to the instruction manual. Specially, the VEGF ELISA kit recognizes all the isoforms of VEGF.

**Gelatin Zymography for Activity of MMP-2 and MMP-9**

Gelatin zymography was used to detect the protein of MMP-2 and MMP-9 expressed in RPE-choroid-sclera complexes 3 days after laser injury. Zymography was performed as described by Munaut et al.24 with some modifications. Briefly, 30 $\mu$L homogenate supernatant of the RPE-choroid complex (contained 30 $\mu$g total protein, determined by DC protein assay kit; Bio-Rad Laboratories) was added to each lane and electrophoresed in 10% sodium dodecyl sulfate (SDS)-polyacrylamide with 1 mg/mL gelatin (Sigma). The gels were soaked in wash buffer (Triton; Sigma), then stained overnight with Coomassie brilliant blue and destained with methanol and acetic acid. Zones of clearing in the gel indicated MMP activity, and the gray intensity value of the proteolytic zone of the gels compared with those of the PBS control group were calculated (ImageQuant software, version 5.2; Molecular Dynamics, Sunnyvale, CA).

**Real-Time PCR for mRNA Expression of MMP-2**

Total RNA was isolated (Trizol; Invitrogen) from the RPE-choroid complexes 3 days after photocoagulation. RPE-choroid complexes

\[
\begin{align*}
&\text{OH} \\
&\text{O} \equiv \text{P} \equiv \text{O} \\
&\text{H} \cdots \text{H} \cdots \text{H} \\
&\text{OH} \\
&\text{O} \equiv \text{P} \equiv \text{O} \\
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&\text{OH} \\
&\text{O} \equiv \text{P} \equiv \text{O} \\
&\text{H} \cdots \text{H} \cdots \text{H} \\
&\text{OH}
\end{align*}
\]

**Figure 1.** Chemical structure of $^{99}$Tc-MDP.
from both eyes of a mouse were pooled for analysis as the sample for this mouse (five mice in each group). Yield and purity were determined photometrically. Complementary DNA synthesis was performed with a first-strand DNA synthesis kit (Quantiscript; Qiagen, Valencia, CA) and then was quantified by real-time PCR analysis (ABI7500; Applied Biosystems, Carlsbad, CA). Sequences of the PCR primers were as follows: (MMP-2) forward primer, 5’-GGCCCATGATGCTTG-3’; reverse primer, 5’-TTGGATTGGTCCTGGGA-3’; probe, 5’-FAM-TGGCAATGCAGATGGACAGCCC-TAMRA-3’; (β-actin) forward primer, 5’-GGCGGCTACAGCTTCA-3’; reverse primer, 5’-TCTCTTATGTCACGCACGAT-3’; probe, 5’-FAM-TGGCAATGCAGATGGACAGCCC-TAMRA-3’. The PCR mix was denatured at 95°C for 3 minutes, followed by 40 cycles of melting at 93°C for 15 seconds, annealing at 55°C for 32 seconds, and elongation at 72°C for 32 seconds. The data were normalized relative to β-actin mRNA levels.

**In Vitro Assays**

**Proliferation and Cytotoxicity of Cells.** Effects of 99Tc-MDP on the proliferation of RF/6A cells (rhesus macaque choroid-retinal endothelial cell; purchased from the Institute of Biochemistry and Cell Biology, Shanghai, China) were estimated by bromodeoxyuridine (BrdU) assay. Cells were seeded into 96-well plates with a density of 1 × 10^4 cells/well. Blank control (wells without cells) and background (wells with cells but without 99Tc-MDP treatment) were used. One day after plating (cells were still nonconfluent and dividing at this time), the medium was removed and the cells were incubated with 99Tc-MDP at the doses of 0.5 × 10^-1 μg/kg BW (n = 5), 1 × 10^-1 μg/kg BW (n = 5), 2 × 10^-1 μg/kg BW (n = 5), or the same volume of PBS (n = 5). The mice were kept in the dark environment for 12 hours and were prepared under dim red illumination, and then flash ERG recordings were performed in the right eye (RETopt; Roland Consult, Brandenburg, Germany), as previously described. Briefly, mice were anesthetized and were placed on a heating pad with a constant temperature of 39°C throughout the experiment. Pupils were dilated with 0.5% tropicamide-phenylephrine ophthalmic solution (Mydrin; Santen Pharmaceutical, Osaka, Japan), and corneas were anesthetized with a drop of 0.5% proparacaine hydrochloride (Alcon, Puurs, Belgium). Custom gold wire electrodes were placed on the corneas. Reference and ground electrodes were placed subcutaneously between the eyes and inserted into the tails, respectively. Eyes were wiped with a cotton bud, and then the remaining filters with the migrated cells were mounted on a glass slide. Four randomly chosen fields were photographed per filter (× 20 objective), and the numbers of migrated cells were counted. The experiment was repeated three times.

**Cell Migration Assay.** RF/6A cell migration assay was performed using 50 μg/mL fibronectin (Biological Industries Ltd., Kibbutz Beil Haemek, Israel)-coated, 24-well permeable cell inserts (Boyden chamber, Transwell; Corning Costar, Corning, NY) with 8-μm pore size, as described previously with some modifications. Briefly, chemotaxis was induced by the addition of 800 μL Dulbecco’s modified Eagle medium (DMEM) with 0.1% bovine serum albumin (BSA; Sigma), with or without 10 ng/mL VEGF (R&D Systems Inc.) to the lower compartment. Serum-free DMEM (200 μL) with 0.1% BSA of 1 × 10^6 RF/6A cells per well were placed in the upper compartment with or without 99Tc-MDP (0.5 × 10^-1 μg/mL, 0.25 × 10^-1 μg/mL, or 0.125 × 10^-1 μg/mL). The transwell units were then incubated at 37°C (95% air/5% CO2) for 6 hours. The filters were fixed and stained with hematoxylin; nonmigrated cells (on top of the filters) were wiped with a cotton bud, and then the remaining filters with the migrated cells were mounted on a glass slide. Four randomly chosen fields were photographed per filter (× 20 objective), and the numbers of migrated cells were counted. The experiment was repeated three times.

**Results**

**Suppression of CNV in Mice Receiving 99Tc-MDP**

Seven days after laser photocoagulation, CNVs on RPE-choroid-sclera flat mounts examined by fluorescence microscopy showed that 99Tc-MDP significantly suppressed the development of CNV in a dose-dependent manner (Fig. 2). 99Tc-MDP treatment of 0.5 × 10^-1 μg/kg (5698.60 ± 1037.70 μm², P < 0.001; n = 13), 1 × 10^-1 μg/kg (3678.34 ± 1328.18 μm², P < 0.001; n = 13), and 2 × 10^-1 μg/kg (2365.78 ± 923.80 μm², P < 0.001; n = 13) could respectively suppress the CNV areas by 36.12%, 58.76%, and 73.48% compared with PBS treatment group (8920.36 ± 1097.29 μm²; n = 13).

**Inhibitory Effects of 99Tc-MDP on Macrophage Infiltration**

We quantitated the infiltration of macrophages in CNV with an immunofluorescence method. The numbers of macrophages per
laser lesion were calculated to determine whether $^{99}$Tc-MDP had an inhibitive effect on the macrophage infiltration. Immunofluorescence intensity for F4/80 tended to be lower in the $^{99}$Tc-MDP–treated mice than in the PBS-treated mice (Fig. 3A), and $^{99}$Tc-MDP had the ability to decrease the numbers of infiltrating macrophages in a dose-dependent manner ($n = 11–12$) (Fig. 3B).

### Inhibition of VEGF and Inflammatory Molecules by the Treatment of $^{99}$Tc-MDP

Macrophages invaded the site of laser injury within 1 day, and peak macrophage numbers paralleled the maximal amount of VEGF protein detected in the RPE-choroid complexes 3 days after laser photoocoagulation. Therefore, to determine whether the protein level of VEGF and inflammatory molecules had the parallel relationship with the numbers of infiltrating macrophages, we collected RPE-choroid complexes 3 days after laser photoocoagulation and detected protein levels of VEGF, ICAM-1, and TNF-α in the RPE-choroid complex by using ELISA. The results showed that $^{99}$Tc-MDP treatment significantly suppressed protein levels of VEGF ($n = 7–8$), ICAM-1 ($n = 12$), and TNF-α ($n = 12$) compared with the PBS treatment group ($P < 0.05$ for all) (Figs. 3C-E). However, for VEGF, there was no statistical significance between the $1 \times 10^{-1} \mu$g/kg BW group and the $2 \times 10^{-1} \mu$g/kg BW group ($P > 0.05$) or between the $1 \times 10^{-1} \mu$g/kg BW group and the $0.5 \times 10^{-1} \mu$g/kg BW group ($P > 0.05$). For ICAM-1, there was no statistical significance between the $1 \times 10^{-1} \mu$g/kg BW group and the $2 \times 10^{-1} \mu$g/kg BW group ($P > 0.05$). For TNF-α, although there was statistical significance between the $^{99}$Tc-MDP–treated groups and the PBS-treated group ($P < 0.01$), there was no statistical significance between any two of the $^{99}$Tc-MDP–treated groups ($P > 0.05$).
**Figure 3.**

(A) Immunofluorescence of F4/80 (green) and PECAM-1 (red) in liver tissue sections from mice treated with different doses of 
$^{99}$Tc-MDP ($\times 10^{-1}$μg/kg BW) compared to PBS control. Scale bars: 20 μm.

(B) Number of macrophages (per laser lesion) in liver tissue sections from mice treated with different doses of 
$^{99}$Tc-MDP ($\times 10^{-1}$μg/kg BW) compared to PBS control.

(C) VEGF expression (pg/mg total protein) in liver tissue sections from mice treated with different doses of 
$^{99}$Tc-MDP ($\times 10^{-1}$μg/kg BW) compared to PBS control.

(D) ICAM-1 expression (pg/mg total protein) in liver tissue sections from mice treated with different doses of 
$^{99}$Tc-MDP ($\times 10^{-1}$μg/kg BW) compared to PBS control.

(E) TNF-α expression (pg/mg total protein) in liver tissue sections from mice treated with different doses of 
$^{99}$Tc-MDP ($\times 10^{-1}$μg/kg BW) compared to PBS control.
Inhibition of MMP-2 Activity by Zymography

Gelatin zymography is a sensitive technique that detects activated gelatin-degrading metalloproteinases such as MMP-2 and MMP-9. Cultured supernatant of the human HT1080 cell was used as a molecular weight marker. The gray intensity value of the proteolytic zone of the gels in the 99Tc-MDP-treated group was significantly lower than that in the PBS control group for active MMP-2 (**P < 0.01; n = 9) but not for pro-MMP-9 (P > 0.05; n = 9), as is shown in Figure 4.

Inhibition of mRNA Expression of MMP-2 by Real-Time PCR

Real-Time PCR was used to determine MMP-2 mRNA expression in RPE-choroid complexes 3 days after photocoagulation.
The results showed that 99Tc-MDP treatment had a significantly concentration of 99Tc-MDP (0.05 μg/mL) was attracted by 10 ng/mL VEGF in a concentration-dependent group (without VEGF in the culture medium). 99Tc-MDP approximately 100% migration activity compared with the basal group.

In the cell migration assay, as is shown in Figures 8A and 8B, in vitro inhibition of VEGF-induced migration by 99Tc-MDP was cytotoxic only to dividing cells. In the BrdU assay we used dividing (not confluent) cells, which represented the pathologic condition of active CNV to some extent, to determine the antiproliferative effect of 99Tc-MDP. First, 99Tc-MDP significantly suppressed the development of CNV in murine models in vivo (Fig. 2) and had an antiproliferative effect on the RF/6A cell in vitro in a dose-dependent manner (Fig. 7A). Second, 99Tc-MDP inhibited VEGF-induced RF/6A cell migration and tube formation in vitro (Figs. 8, 9), both in a dose-dependent manner. Third, 99Tc-MDP treatment decreased the numbers of macrophages infiltrating the CNV (Figs. 3A, 3B), together with downregulated protein expression of VEGF (Fig. 3C) and inflammatory molecules including ICAM-1 and TNF-α (Figs. 3D, 3E). In addition, 99Tc-MDP treatment inhibited the mRNA expression and gelatin-degrading activity of MMP-2 (Figs. 4, 5). Importantly, electroretinography and cytotoxicity study showed that any dose of 99Tc-MDP used in our study was not toxic to the mouse retina (Fig. 6) or to nondividing (confluent) cells (Fig. 7B).

**Discussion**

In this present study, our data for the first time revealed several important findings concerning the antiangiogenic effects of 99Tc-MDP on the development of CNV with its anti-inflammatory property. First, 99Tc-MDP significantly suppressed the development of CNV in murine models in vivo (Fig. 2) and had an antiproliferative effect on the RF/6A cell in vitro in a dose-dependent manner (Fig. 7A). Second, 99Tc-MDP inhibited VEGF-induced RF/6A cell migration and tube formation in vitro (Figs. 8, 9), both in a dose-dependent manner. Third, 99Tc-MDP treatment decreased the numbers of macrophages infiltrating the CNV (Figs. 3A, 3B), together with downregulated protein expression of VEGF (Fig. 3C) and inflammatory molecules including ICAM-1 and TNF-α (Figs. 3D, 3E). In addition, 99Tc-MDP treatment inhibited the mRNA expression and gelatin-degrading activity of MMP-2 (Figs. 4, 5). Importantly, electroretinography and cytotoxicity study showed that any dose of 99Tc-MDP used in our study was not toxic to the mouse retina (Fig. 6) or to nondividing (confluent) cells (Fig. 7B).

**Proliferation and Cytotoxicity of Cells In Vitro**

In the BrdU assay we used dividing (not confluent) cells, which represented the pathologic condition of active CNV to some extent, to determine the antiproliferative effect of 99Tc-MDP. The results showed that 99Tc-MDP treatment had a significantly inhibitive effect on cell proliferation in a dose-dependent manner (Fig. 7A) ($P < 0.001$; $n = 6$). However, 99Tc-MDP had no influence on completely confluent (nondividing) cells in the MTT assay (Fig. 7B) ($P > 0.05$; $n = 8$). The data suggested that 99Tc-MDP was cytotoxic only to dividing cells.

**In Vitro Inhibition of VEGF-Induced Migration and Tube Formation of RF/6A Cells**

In the cell migration assay, as is shown in Figures 8A and 8B, stimulation by 10 ng/mL VEGF resulted in an increase of approximately 100% migration activity compared with the basal group (without VEGF in the culture medium). 99Tc-MDP (0.0125–0.05 μg/mL) significantly inhibited cell migration chemotactically by 10 ng/mL VEGF in a concentration-dependent manner ($P < 0.001$; $n = 9$) (Figs. 8C-F). At the highest concentration of 99Tc-MDP (0.05 μg/mL), the number of migrated cells was suppressed by 76.78% compared with the PBS control group.

In the VEGF-induced capillary-like vessel formation assay, VEGF significantly enhanced tube formation (Figs. 9A, 9B). At the concentration of 0.025 μg/mL 99Tc-MDP, the length of the formed capillary-like vessel was suppressed to the basal group level (without VEGF in the culture medium) (Figs. 9A, 9D, 9E). Figures 9C to 9E showed a decreasing tendency of annular structures and numbers of branch points of capillary-like vessels, especially in the 0.05-μg/mL 99Tc-MDP-treated group compared with the PBS control group. Vessel lengths were suppressed by the treatment of 99Tc-MDP in a concentration-dependent fashion (Fig. 9F) ($P < 0.001$; $n = 12$).

These results demonstrated that 99Tc-MDP had the ability to block VEGF-induced angiogenesis in vitro.
Angiogenesis is the formation of new blood vessels from preexisting vasculature. As we know, the well-defined steps of angiogenesis include extracellular matrix (ECM) degradation, endothelial cell migration, cell proliferation, tube formation, and vessel wall remodeling. Migration and tube formation of endothelial cells have been thought to be important steps in angiogenesis.

Our in vitro experimental data had shown that 99Tc-MDP inhibited not only cell proliferation but also VEGF-induced cell migration and tube formation in a dose-dependent manner (Figs. 7A, 8, 9), which could be an important cellular mechanism for the inhibitive effects of 99Tc-MDP on the CNV in vivo murine model.

As discussed, ECM degradation is the initial step for endothelial cell migration. ECM degradation, which facilitates endothelial cell migration further into the subretinal space during angiogenesis, is thought to be a very important step in angiogenesis, and this process is potentiated by the secretion of matrix metalloproteinases (MMPs). Among MMPs, MMP-2 and MMP-9 are particularly noted by researchers because they preferentially degrade type IV collagenase (the main component of the basement membrane). Mice with MMP-2 and MMP-9 gene deficiency have a decreased incidence and severity of laser-induced CNV. Our data showed that MMP-2 mRNA expression was upregulated in the PBS control group (with laser photocoagulation) compared with the non-laser-treated mouse group (basal group), and 99Tc-MDP had the property of inhibiting MMP-2 mRNA expression in a dose-dependent manner (Fig. 5). Results of gelatin-degrading activity of MMP-2 paralleled the real-time PCR results (Figs. 4, 5), and these results suggested that decreased gelatin-degrading activity of MMP-2 might be related to the reduced mRNA expression of MMP-2. Because MMP-2 can be directly secreted by macrophages and MMP-2 mRNA expression can be upregulated by VEGF, based on our data we propose that the inhibitive effects of 99Tc-MDP on the mRNA expression of MMP-2, which directly leads to the decreased gelatin-degrading activity of MMP-2, are due to the suppression of infiltrating macrophages and the inhibited protein expression of VEGF.

Increasing evidence has supported the notion that inflammatory mechanisms play a role in the pathogenesis of CNV. As one of the most important inflammatory cells, macrophages play a critical role in the development of CNV. By secreting growth factors and inflammatory cytokines, macrophages can influence every phase of the angiogenic process, such as alterations of the local extracellular matrix, migration, and proliferation of endothelial cells. Blood-derived macrophages infiltrating the CNV are thought to be a rich source of VEGF. Generalized macrophage depletion reduced the size and leakage of laser-induced CNV associated with decreased macrophage infiltration and VEGF protein expression. The process of angiogenesis is controlled by a variety of cells and molecular mediators. Macrophages can promote angiogenesis, and this promotive effect is thought to be related to its production of VEGF and a variety of inflammatory cytokines, such as ICAM-1, TNF-α, and MMPs. Importantly, ICAM-1 is expressed on RPE and the vascular endothelial cell surface and is an important component of cell-to-cell interactions during inflammatory responses, mediating leukocyte (including macrophages) adhesion. The expression of ICAM-1 in cultured human RPE cells is upregulated by the stimulation of IL-1β and TNF-α. TNF-α is a pleiotropic cytokine that mediates inflammatory, proliferative, and cytotoxic effects in a variety of cell types. TNF-α contributes to the development of choroidal neovascularization through triggering VEGF production by RPE cells. In addition, TNF-α enhances the expression of other angiogenic regulators such as MCP-1 and interleukins. Based on these facts, we can see there is a closed but amplifying circuit among VEGF, inflammatory

### Table 1. Amplitudes and Implicit Times of Pre-99Tc-MDP Treatment and Post-99Tc-MDP Treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>a-Wave Amplitudes (µV, n = 5)</th>
<th>b-Wave Amplitudes (µV, n = 5)</th>
<th>b-Wave Implicit Times (ms, n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS-treated group</td>
<td>Pretreatment 107 ± 27.1</td>
<td>Posttreatment 108 ± 29.4</td>
<td>114 ± 29.0</td>
</tr>
<tr>
<td>1 × 10⁻³ µg/kg BW</td>
<td>104 ± 28.9</td>
<td>106 ± 29.0</td>
<td>112 ± 30.0</td>
</tr>
<tr>
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<td>103 ± 29.0</td>
<td>107 ± 28.9</td>
<td>114 ± 29.0</td>
</tr>
<tr>
<td>5 × 10⁻³ µg/kg BW</td>
<td>102 ± 28.9</td>
<td>106 ± 29.0</td>
<td>112 ± 30.0</td>
</tr>
<tr>
<td>10⁻³ µg/kg BW</td>
<td>101 ± 28.9</td>
<td>105 ± 29.0</td>
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<td>101 ± 29.0</td>
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<tr>
<td>5 × 10⁻³ µg/kg BW</td>
<td>96 ± 28.9</td>
<td>100 ± 29.0</td>
<td>106 ± 29.0</td>
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</tbody>
</table>

Data are expressed as the mean ± SD. *P < 0.05, t-test statistic.
cells (especially for macrophages), and inflammatory cytokines. The initial objective of this study was to use the anti-inflammatory property of 99Tc-MDP to interrupt this inflammatory cascade circuit to inhibit CNV formation. Our cellular and molecular data showed that 99Tc-MDP can inhibit macrophages infiltrating to the CNV and the downregulated expression of VEGF, ICAM-1, and TNF-α. Based these data, we put forward a hypothesis for the mechanisms of inhibiting the effect of 99Tc-MDP on CNV as follows. First, 99Tc-MDP decreases the number of macrophages infiltrating the CNV. Macrophages are the main sources of VEGF, which is a critical angiogenic factor in CNV formation; therefore, the production

![Figure 7](image-url)

**FIGURE 7.** Antiproliferative effects of 99Tc-MDP on dividing RF/6A cells by BrdU assay and cytotoxicity of 99Tc-MDP on nondiving RF/6A cell viability by MTT assay. (A) BrdU assay data revealed that 99Tc-MDP treatment had a significantly inhibitive effect on cell proliferation in a dose-dependent manner. (**P < 0.001 compared with the PBS control group; n = 6). (B) Percentage data compared with PBS control group were expressed as mean ± SD in the MTT assay. 99Tc-MDP had no influence on completely confluent cells (nondividing cells) compared with the PBS-treated mice (P > 0.05; n = 8).

![Figure 8](image-url)

**FIGURE 8.** 99Tc-MDP inhibited VEGF-induced cell migration in vitro. We used 8-μm pore sized transwell inserts in the cell migration study. The photographs were taken (×20 objective) 6 hours after transwell inserts incubated in a 37°C incubator. (A) Basal group without VEGF or 99Tc-MDP. (B–E) All received 10 ng/mL VEGF as the chemoattractants. (C–E) Received increasing doses of 99Tc-MDP (0.0125, 0.025, and 0.05 μg/mL in (C), (D), and (E), respectively. and (B) Received PBS as the control group. (F) ANOVA revealed that each of the 99Tc-MDP treatment groups had statistically significant differences compared with the PBS treatment group (**P < 0.001; n = 9), and the numbers of the migrated cells were significantly inhibited by 99Tc-MDP in a concentration-dependent manner. Scale bars, 50 μm (A–E).
of VEGF protein will be reduced. Second, reductions in the release of TNF-α due to the decreased numbers of macrophages can weaken ICAM-1 expression in endothelial cells and RPE cells, which will mediate fewer macrophages adhering to the CNV area. As a result, less VEGF protein will be produced, and CNV development will be suppressed. In addition, the in vitro experiments showed that 99Tc-MDP could affect the downstream signaling transduction that stimulates the proliferation, migration, and tube formation of endothelial cells after VEGF binding to its receptors. Therefore, 99Tc-MDP not only reduces VEGF expression in vivo (Fig. 3C), it also blocks the function of already existing VEGF in vitro (Figs. 8, 9). This multiple targeting sites of 99Tc-MDP on VEGF may partially explain the inhibitive effects of 99Tc-MDP on the development of CNV, though the exact mechanisms have not been disclosed completely.

The 99Tc-MDP used in our study is different from the agent 99mTc-MDP (a radioactive imaging agent widely used for bone scintigraphy). Actually, 99Tc-MDP is the decay product of 99mTc-MDP. 99mTc-MDP has little radioactivity, and this small amount of radioactivity has been demonstrated to be harmless to the human body in prolonged systemic administration in the clinical treatment of systemic inflammatory diseases such as RA and AS in China. Because of its powerful anti-inflammatory effect and its safety, we chose 99Tc-MDP but not 99mTc-MDP for our experiment. Significantly, electoretinography and cytotoxicity showed that any dose of 99Tc-MDP used in our study was not toxic to the mouse retina (Fig. 6) or to non-dividing (confluent) cells (Fig. 7B). Furthermore, in our in vivo study, we found no body weight changes (data not shown) or mouse deaths caused by the injection of 99Tc-MDP. Based on these facts, we believe that 99Tc-MDP is a safe and effective anti-inflammatory agent.

In summary, our experimental data showed that 99Tc-MDP can affect multiple inflammatory steps during CNV development, including MMP-2–mediated extracellular matrix degrada-
tion, recruitment of macrophages mediated by ICAM-1, and downregulated expressions of VEGF and inflammatory cytokines derived from macrophages. However, though 99Tc-MDP reduces CNV formation significantly, the incidence rate of CNV between the 99Tc-MDP treatment groups and the PBS treatment group are of no statistical significance (data not shown), suggesting that mechanisms other than inflammatory ones are involved in the angiogenesis of laser-induced CNV. In addition, as was shown in Figures 7A and 7B, 99Tc-MDP had a specific inhibiting effect only on proliferating cells but not on non-proliferating cells, suggesting that 99Tc-MDP might help to inhibit those active CNV lesions. However, pathologic mechanisms of CNV murine models are different from those of human beings. Therefore, the effects of 99Tc-MDP on human beings still must be confirmed in clinical studies.

All these findings support the idea that inflammatory mechanisms play an important role in the pathogenesis of CNV and suggest that 99Tc-MDP has therapeutic potential to inhibit CNV. Whereas there is still little knowledge about the effects of 99Tc-MDP on CNV, further studies concerning long-term inhibitive effects and more detailed mechanisms of CNV inhibition are needed.

References


