Antiangiogenic Effect of $\text{As}_2\text{O}_3$ on the Angiogenesis Induced by Vascular Endothelial Growth Factor (VEGF) in the Rat Cornea

Do-hyoung Kwon, Jae young Jang, Na-young Yi, Man-bok Jeong, Shin-ae Park, Min-su Kim
Tehi-chou Nam, Myung-jin Park*, Ill-ju Bae**, Chang-hun Rhee* and Kang-moon Seo*

Department of Veterinary Surgery, College of Veterinary Medicine, Seoul National University
*Laboratory of Cell Biology, Department of Neurosurgery, Korea Institute of Radiological and Medical Sciences, Seoul
**Laboratory of Chonjisan Institute, Seoul

Abstract: The purpose of this study was to compare the antiangiogenic effects of $\text{As}_2\text{O}_3$ to those of $\text{As}_2\text{O}_3$ on the rat corneal micropocket model induced by VEGF. 20 ng VEGF impregnated pellets were used for angiogenic inducer on the rat cornea micropocket assay in this study. After ophthalmoscopic examination, Sprague-Dawley rats with normal cornea were implanted VEGF pellet. Total 60 eyes were used in this study. Control group only received VEGF pellet, $\text{As}_2\text{O}_3$ group followed oral administration of $\text{As}_2\text{O}_3$ at a dose of 50 mg/kg per day after VEGF pellet implantation and $\text{As}_2\text{O}_3$ group followed oral administration of $\text{As}_2\text{O}_3$ at a dose of 50 mg/kg per day after VEGF pellet implantation were classified. The eyes were examined under a surgical microscope daily on postoperative from day 3 to day 9 after pellet implantation. The number, length, clock hour of vascularization, and area of vessels in $\text{As}_2\text{O}_3$ group were significantly less evident than those of control group and $\text{As}_2\text{O}_3$ group ($P<0.05$). In conclusion, $\text{As}_2\text{O}_3$ had better antiangiogenic effects on the new vessel induced by VEGF in the rat cornea.

Key words: $\text{As}_2\text{O}_3$, $\text{As}_2\text{O}_3$, VEGF, angiogenesis, rat.

Introduction

Arsenic trioxide ($\text{As}_2\text{O}_3$) is a common, naturally occurring substance. It is rarely found in its pure elemental state in nature\textsuperscript{1,2}. The medical use of arsenic recently is reduced because of its toxicity and potential for carcinogenicity\textsuperscript{3,4}. Although arsenic can be poisonous, and chronic arsenic exposure from natural or industrial sources can cause serious toxicity, arsenic has been used medically for over 2,400 years\textsuperscript{5}. Since the antileukemic activity of arsenic agent was first reported in the late 1800s, the reemerging of arsenic therapy has occurred. In the 1970s, physicians in China began using arsenic trioxide as a part of treatment for acute promyelocytic leukemia (APL)\textsuperscript{6,7,8,9}. Arsenic trioxide was recently used for the treatment of tumors like multiple myeloma\textsuperscript{10}, chronic B-cell leukemia\textsuperscript{11} and acute promyelocytic leukemia.

Although the precise mechanism of $\text{As}_2\text{O}_3$ action is unknown, a variety of in vitro studies suggest that $\text{As}_2\text{O}_3$ contributes to the effectiveness in vivo by some mechanisms including induction of apoptosis, partial cellular differentiation, degradation of specific APL fusion transcripts, antiangiogenesis, and inhibition of angiogenesis\textsuperscript{12,13}. $\text{As}_2\text{O}_3$ interrupted a reciprocal stimulatory loop between leukemic cells and endothelial cells by causing apoptosis of both cell types and by inhibiting leukemic cell vascular endothelial growth factor (VEGF) production, and prevented capillary tubeule and branch formation in an in vitro endothelial cell differentiation assay\textsuperscript{14}.

$\text{As}_2\text{O}_3$ is one of the arsenic compound derivatives, which was used as the empirical anticancer agent in the Korean alternative medicine. However, there were few reports which showed the mechanism of anticancer effect of $\text{As}_2\text{O}_3$. Park et al\textsuperscript{15} suggested that $\text{As}_2\text{O}_3$ significantly decreased the proliferation, migration, invasion, and tube formation of endothelial cells induced by the angiogenic factors bFGF in vitro.

VEGF\textsuperscript{3,6,7,10,11}, basic fibroblast growth factor (bFGF)\textsuperscript{2,3,6,15}, nerve growth factor (NGF)\textsuperscript{16}, epidermal growth factor (EGF)\textsuperscript{8}, and transforming growth factor $\alpha$, $\beta$ are known as the angiogenic positive factors. $\text{As}_2\text{O}_3$ inhibited angiogenic effect of bFGF on the rat corneal micropocket model\textsuperscript{17} and the reverse effects of $\text{As}_2\text{O}_3$ on the angiogenesis induced by NGF in the rat corneal micropocket model\textsuperscript{18}. In addition, Park\textsuperscript{11} reported angiogenic effect of VEGF on the rat corneal micropocket model. However, there have been no studies about antiangiogenic effects of $\text{As}_2\text{O}_3$ induced by VEGF.

The aim of this study was to compare the antiangiogenic effects between the new arsenic compound $\text{As}_2\text{O}_3$ and the existing $\text{As}_2\text{O}_3$ on neovascularization induced by VEGF in the rat micropocket cornea.

Materials and Methods

Experimental animals

Male Sprague-Dawley (SD) rats weighing 300-400 g were used in this study. Animals were housed in cages at a controlled temperature (22±2°C, 55±10% humidity) with a 12 h light/dark cycle, and fed with commercial rat pellet, and free access to water at all times throughout the study. The rats were acclimatized for 7 days prior to commencement of...
experiments. All animal experimentation was performed in accordance with guideline for the use and care of laboratory animal in Seoul National University. After ophthalmascopic examination, rats with normal cornea only were performed VEGF pellet implantation. Total 60 eyes were used in this study.

Experimental design

The entire groups were randomly divided into three groups; control group only received VEGF pellet, As_<sub>2</sub>O_<sub>3</sub> group followed the trans-oral administration of As_<sub>2</sub>O_<sub>3</sub> at a dose of 50 mg/kg per day after VEGF pellet implantation, and As_<sub>2</sub>O_<sub>3</sub> group followed the trans-oral administration of As_<sub>2</sub>O_<sub>3</sub> at a dose of 50 mg/kg per day after VEGF pellet implantation.

VEGF pellet preparation

Pellets were prepared according to the method previously described.<sup>4,14,15,23</sup> Sterile casting solution was prepared by dissolving the poly-2-hydroxyethylmethacrylate (Hydon, Sigma Co, USA) powder in absolute ethanol (12% w/v) at 37°C with continuous stirring for 24 hrs. An equal volume of Hydon and sucralfate (12% w/v, Sigma Co, USA) were combined because the Hydon can stabilize the VEGF and sucralfate induces slow release of VEGF. Both materials do not affect the angiogenic response. Then 20 ng of VEGF were mixed with 2 μl of Hydon and sucralfate combined solution. These solutions were pipetted onto the surface of sterile teflon rods glued to the surface of petri dish to make a pellet with diameter of 2 mm. Pellets were dried at room temperature for 1 to 2 hrs in sterile environment, then stored at -20°C until used.

VEGF pellet implantation

VEGF pellet implantation into rat corneas was conducted by the method previously described.<sup>4,14,15,23</sup> Rats were anesthetized with a combination of ketamine hydrochloride (20 mg/kg, IM, Yuhan Ketamine®, Yuhan Co., Korea) and xylazine hydrochloride (6 mg/kg, IM, Rompun®, Bayer Co., Korea). The eyes were topicaly anesthetized with 0.5% proparacaine (Alcaine®, Alcon Co., USA), and gently proposted and secured by clamping the upper eyelid with a nontraumatic hemostat. Under a surgical microscope, 1.5 mm incision was made approximately 1 mm from the center of cornea into stroma with Beaver's blade (Beaver™, Becton Dickinson Co., USA) but not through it. A microdissector approximately 1.5 mm in width was inserted under the lip of the incision and gently blunt-dissected through the stroma toward limbus of the eye. Slight finger pressure against the globe of the eye was kept steady it during dissection. The distance between the limbus and base of the pocket was kept 1-1.5 mm in length. The VEGF pellet was rehydrated with a drop of sterile saline. The pellet was positioned down to the base of the pocket should be occupied with the implant material. After pellet implantation, antibiotic ointment (Terramycin®, Pfizer Co., Korea) was pasted around rat cornea and rat was marked on its tail.

Biomicroscopic examination

The eyes were examined under a surgical microscope (Leica M651) daily on postoperative from day 3 to day 9 after pellet implantation. Images were captured using a digital camera. The number of vessels, the length of vessels, and the angle of neovascularization were evaluated using image analyzing program (Image tools ver 3.0, the University of Texas Health Science Center in San Antonio, USA). The contiguous circumferential zone of neovascularization was measured as clock hours with a 360° reticule (where 30° of arc equals 1 clock hour). The area of neovascularization was determined by measuring with a reticule the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. A formula was used to determine the area of a circular band segment: C/12 × 3.1416 [r²-(r-L)²], where r = 2.5 mm, the measured radius of the rat cornea.<sup>5</sup>

Statistical analysis

For a intention verification of this study, all numerical variables (number of vessels, length of vessels, clock hour of neovascularization, and area of neovascularization) were assessed by use of repeated ANOVA test with two factors; time and treatment (control, As_<sub>2</sub>O_<sub>3</sub>, As_<sub>2</sub>O_<sub>3</sub>). And their interactions were examined. When significant difference was determined, multiple comparisons at each time point were made using Scheffe test. Values of P <0.05 were considered significant.

Results

The number of vessels

New vessels began sprouting into the cornea on postoperative day 3. The number of vessels increased in all groups with time. The number of vessels in As_<sub>2</sub>O_<sub>3</sub> group were significantly fewer than control group and As_<sub>2</sub>O_<sub>3</sub> group from day 3 to day 6 (P <0.05). However, there was no significant difference of the vessel number in As_<sub>2</sub>O_<sub>3</sub> group and As_<sub>2</sub>O_<sub>3</sub> group after day 7. They were significantly fewer than control group as ever (Fig 1).

The length of vessels

Vessel length changes in each group showed a similar pattern to the number of vessels. The length of vessels in As_<sub>2</sub>O_<sub>3</sub> group was significantly shorter than that of control group and As_<sub>2</sub>O_<sub>3</sub> group (P <0.05). The vessel length in As_<sub>2</sub>O_<sub>3</sub> group was somewhat longer than control group, but there is little point in it (Fig 2).

The clock hours of neovascularization

Clock hour changes of neovascularization in each group showed a growth pattern that was similar to that of the length of vessels. As_<sub>2</sub>O_<sub>3</sub> group showed significantly narrower clock hours of neovascularization than in control group and As_<sub>2</sub>O_<sub>3</sub> group from day 3 to day 7 (P <0.05; Fig 3).
Fig 1. Change of the number of vessels after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

Fig 2. Change of the length of vessels after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

Fig 3. Change of the the clock hours of neovascularization after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

Fig 4. Change of the area of neovascularization after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

Area of neovascularization

As$_2$O$_3$ group was found to have significantly lesser vessel area than in control group and As$_2$O$_5$ group from day 3 to day 9 ($P<0.05$; Fig 4).

Discussion

The purpose of this study was to compare the antiangiogenic effects of As$_2$O$_3$ to those of As$_2$O$_5$ on the rat corneal micropocket model induced by VEGF. With application of cornea micropocket assay, the confusion could be avoided between new vessels and previously exiting vessels.

20 ng VEGF impregnated pellets were used for angiogenic inducer on the rat cornea micropocket assay in this study. Park$^{23}$ used the same dose as the optimal effective dose of VEGF, and reported that the dose did not induce side effects such as corneal edema or inflammation after operation. We also found VEGF had no significant side effects in rats at the above same dose.

This study showed that As$_2$O$_3$ inhibited angiogenesis induced by VEGF in the rat cornea. As$_2$O$_3$ had a better antiangiogenic activity than As$_2$O$_5$ on VEGF. Park et al$^{23}$ and You et al$^{23}$ reported that As$_2$O$_5$ had a better antiangiogenic effects on the neovascularization induced by bFGF and NGF in the rat cornea. They suggested that there was different antiangiogenic mechanism between As$_2$O$_3$ and As$_2$O$_5$. In contrast to the severe gastrointestinal toxicities of oral administration of As$_2$O$_3$, they also observed that oral administration of As$_2$O$_3$ at the dose of 50 mg/kg per day for 7 days to SD rats and for 22 days to C57BL/6 mice caused no remarkable side effects. In this study, we also found As$_2$O$_3$ had no noticeable side effects in SD rats at the above same dose for 9 days. The oral rat LD$_{50}$ of As$_2$O$_3$ for investigation as mutagen, tumorigen and reproductive effector was known as 14.6 mg/kg.
Fig 5. Appearance of angiogenesis on day 9 after VEGF pellet implantation on the rat corneal stroma and administration of arsenic compounds per oral. A. control group. B. As$_2$O$_3$ group. C. As$_5$O$_6$ group. "E": New vessels.

VEGF has reported to have an endothelial cell-specific mitogen and an angiogenesis inducer released by a variety of tumor cells and expressed in human tumors in situ. Angiogenic effect of VEGF in this study is in agreement with the Kenyon's research who observed angiogenesis at the limbus of rat cornea 5 to 6 days after post-implantation using VEGF.

Lee et al$^9$ reported that VEGF had the dose-dependent relationship between VEGF-dose and matrix metalloproteinase (MMP) -2, 9 (gelatinase) in rat cornea. Park et al$^{10}$ demonstrated that As$_2$O$_3$ appeared to be a novel antiangiogenesis and antimetastasis chemical agent that can be orally administered in animal models. They also observed that induction of cell cycle arrest at G$_2$/M phase and inhibition of MMP-2 secretion might be possible antiangiogenic mechanisms of As$_2$O$_3$. In this study, As$_2$O$_3$ had better antiangiogenic effect than As$_5$O$_6$ in length, and area of vessels. As As$_5$O$_6$ inhibited the angiogenesis induced by VEGF, it was hypothesized that As$_2$O$_3$ might suppress autocrine secretion.

In recent years, arsenic trioxide (As$_2$O$_3$) has been used successfully in the treatment of patients with acute promyelocytic leukemia (APL). Some recent studies showed antiangiogenic activity of As$_2$O$_3$. As$_2$O$_3$ acts on cells through a variety of mechanisms as influencing numerous signal transduction pathways and resulting in a vast range of cellular effects that include apoptosis induction, growth inhibition, promotion or inhibition of differentiation, and angiogenesis inhibition.$^{12}$

Roboz et al$^{17}$ reported that As$_2$O$_3$ inhibited capillary tube and branch formation in an in vitro endothelial cell-differentiation assay. They believed that As$_2$O$_3$ interrupted a reciprocal stimulatory loop between leukemic cells and endothelial cells by causing apoptosis of both cell types and by inhibiting leukemic cell VEGF production. It is known that impaired production or expression of VEGF will disrupt angiogenesis.$^{17}$

As$_2$O$_3$ significantly decreased the proliferation, migration, invasion, and tube formation of endothelial cells induced by the angiogenic factors bFGF. As$_2$O$_3$ also inhibited angiogenesis in the rat corneal micropocket model induced by bFGF and lung metastasis in the mouse model in vivo and migration and invasion of bovine capillary endothelial (BCE) cells, and tubular structure formation of human umbilical vein endothelial cells (HUVECs) on Matrigel.$^{15}$ As$_2$O$_3$ also had antiangiogenic effects on the new vessels induced by NGF in the rat cornea. However there are almost never antiangiogenic effects in As$_5$O$_6$ group.$^{25}$

The different angiogenic factors may control the formation of new vessels via either similar or distinct signaling pathways in angiogenesis. There were a total of 94 human genes with differential expression patterns in response to mitogen treatment. And the thirty-two gene expression patterns in 94 genes were similarly regulated by either VEGF or bFGF, whereas those of the remaining sixty-two genes were regulated by only one of them$^9$.

It may be suggested that there were different mechanisms between As$_2$O$_3$ and As$_5$O$_6$ in antiangiogenic activity and As$_5$O$_6$ had more antiangiogenic effects than As$_2$O$_3$ in similar signaling pathways.

**Conclusion**

To evaluate the antiangiogenic effects of As$_2$O$_3$ and As$_5$O$_6$ on the angiogenesis induced by VEGF, cornea micropocket assay in the rat was used. The number, length, the clock hour of vascularization, and the area of the vessels in As$_2$O$_3$ group were significantly less evident than those of control group and As$_5$O$_6$ group during experimental period (P < 0.05). In conclusion As$_2$O$_3$ had better antiangiogenic effects on the new vessel induced by VEGF in the rat cornea.

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랜드 간막에서 Vascular Endothelial Growth Factor (VEGF)로 유발시킨 신생혈관에 대한 As₄O₆의 혈관신생 억제효과

권도형 · 장재영 · 이나영 · 정만복 · 박신애 · 김민수 · 남처주 · 박명진* · 박은주** · 이창훈* · 서강문
서울대학교 의과대학
*원자력병원, 신경외과, 분자생물학연구실
**서울중앙대병원, 임상의학

요 약 : 본 연구는 VEGF로 유발된 랜드 간막 미세혈 모질에서 As₄O₆와 As₂O₃의 혈관신생 억제효과를 비교하기 위해 실시하였다. 20 ng VEGF가 혈관을 채색한 마우스를 이용하여 혈관신생을 유도하였다. 각 약물의 유발자의 특성에 따라 다양한 복합물에 대한 As₂O₃, As₄O₆ 등 혈관신 생 억제 효과가 보였다. 결론적으로, VEGF로 유발된 랜드 간막의 혈관신생 억제 효능을 보여줄 수 있었다.