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Antiangiogenic Effect of As_4O_6 on the Angiogenesis Induced by Vascular Endothelial Growth Factor (VEGF) in the Rat Cornea

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Abstract : The purpose of this study was to compare the antiangiogenic effects of As_4O_6 to those of As_2O_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, As_2O_3 group followed oral administration of As_2O_3 at a dose of 50 mg/kg per day after VEGF pellet implantation and As_4O_6 group followed oral administration of As_4O_6 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 As_4O_6 group were significantly less evident than those of control group and As_2O_3 group ($P<0.05$). In conclusion, As_4O_6 had better antiangiogenic effects on the new vessel induced by VEGF in the rat cornea.

Key words **:** As_4O_6, As_2O_3, VEGF, antiangiogenesis, rat.

Introduction

Arsenic trioxide(As_2O_3) is a common, naturally occurring substance. It is rarely found in its pure elemental state in nature[21]. The medical use of arsenic recently is reduced because of its toxicity and potential for carcinogenicity[1,21]. 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[1]. Since the antileukemic activity of arsenic agent was first reported in the late 1800 s, the reemerging of arsenic therapy has occurred. In the 1970 s, physicians in China began using arsenic trioxide as a part of treatment for acute promyelocytic leukemia (APL)[5,18,19]. Arsenic trioxide was recently used for the treatment of tumors like multiple myeloma[9], chronic B-cell leukemia[12] and acute promyelocytic leukemia.

Although the precise mechanism of As_2O_3 action is unknown, a variety of *in vitro* studies suggest that As_2O_3 contributes to the effectiveness *in vivo* by some mechanisms including induction of apoptosis, partial cellular differentiation, degradation of specific APL fusion transcripts, antiproliferation, and inhibition of angiogenesis[17,21]. As_2O_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 tubule and branch formation in an *in vitro* endothelial cell differentiation assay[17].

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As_4O_6 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 As_4O_6. Park et al[15] suggested that As_4O_6 significantly decreased the proliferation, migration, invasion, and tube formation of endothelial cells induced by the angiogenic factors bFGF *in vitro*.

VEGF[3,6,8,10,16], basic fibroblast growth factor (bFGF)[2,3,6,15], nerve growth factor (NGF)[23], epidermal growth factor (EGF)[8], and transforming growth factor α, β are known as the angiogenic positive factors. As_4O_6 inhibited angiogenic effect of bFGF on the rat corneal micropocket model[15] and the reverse effects of As_4O_6 on the angiogenesis induced by NGF in the rat corneal micropocket model[23]. In addition, Park [14] reported angiogenic effect of VEGF on the rat corneal micropocket model. However, there have been no studies about antiangiogenic effects of As_4O_6 induced by VEGF.

The aim of this study was to compare the antiangiogenic effects between the new arsenic compound As_4O_6 and the existing As_2O_3 on neovascularization induced by VEGF in the rat micropoket 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 ophthalmoscopic 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_2O_3 group followed the trans-oral administration of As_2O_3 at a dose of 50 mg/kg per day after VEGF pellet implantation, and As_4O_6 group followed the trans-oral administration of As_4O_6 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[6,14,15,23]. Sterile casting solution was prepared by dissolving the poly-2-hydroxylethylmethacrylate (Hydron, Sigma Co, USA) powder in absolute ethanol (12% w/v) at 37°C with continuous stirring for 24 hrs. An equal volume of Hydron and sucralfate (12% w/v, Sigma Co, USA) were combined because the Hydron 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 Hydron 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[6,14,15,23]. 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 topically anesthetized with 0.5% proparacaine (Alcaine®, Alcon Co., USA), and gently proptosed 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 detemine the area of a circular band segment: $C/12 \times 3.1416\ [r^2-(r-L)^2]$, where r = 2.5 mm, the measured radius of the rat cornea[2].

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_2O_3, As_4O_6). 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 vessles

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_4O_6 group were significantly fewer than control group and As_2O_3 group from day 3 to day 6 ($P < 0.05$). However, there was no significant difference of the vessel number in As_2O_3 group and As_4O_6 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_4O_6 group was significantly shorter than that of control group and As_2O_3 group ($P < 0.05$). The vessel length in As_2O_3 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_4O_6 group showed significantly narrower clock hours of neovascularization than in control group and As_2O_3 group from day 3 to day 7 ($P < 0.05$; Fig 3).

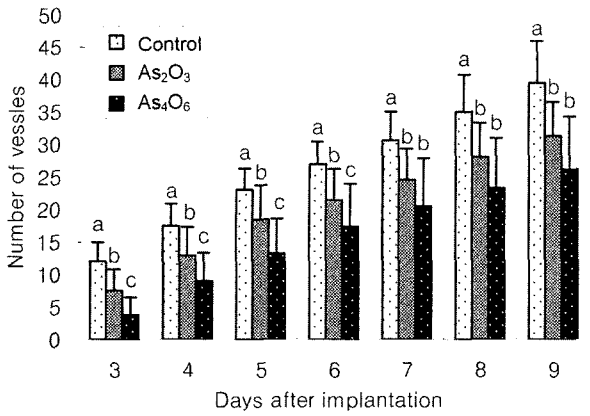

Fig 1. Change of the number of vessels after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

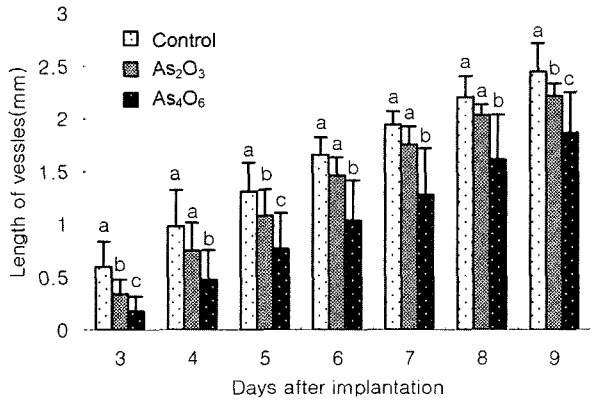

Fig 2. Change of the length of vessels after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

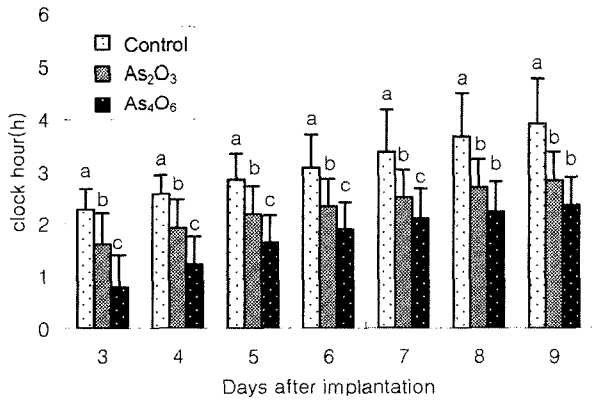

Fig 3. Change of the the clock hours of neovascularization after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c: Different letters within same day mean significantly differences at $P<0.05$.

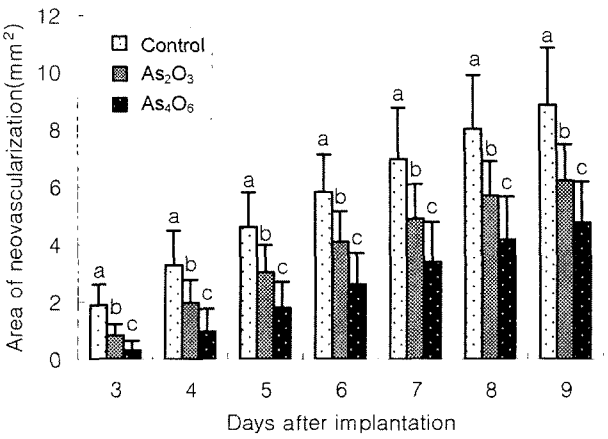

Fig 4. Change of the area of neovascularization after administration of arsenic compound daily in the rat cornea with VEGF pellet implantation. a, b, c : Different letters within same day mean significantly differences at $P<0.05$.

Area of neovascularization

As_4O_6 group was found to have significantly lesser vessel area than in control group and As_2O_3 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_4O_6 to those of As_2O_3 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[10,14,15,23].

20 ng VEGF impregnated pellets were used for angiogenic inducer on the rat cornea micropocket assay in this study. Park[14] 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_4O_6 inhibited angiogenesis induced by VEGF in the rat cornea. As_4O_6 had a better antiangiogenic activity than As_2O_3 on VEGF. Park *et al*[15] and You *et al*[23] reported that As_4O_6 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_2O_3 and As_4O_6. In contrast to the severe gastrointestinal toxicities of oral administration of As_2O_3[18], they also observed that oral administration of As_4O_6 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_4O_6 had no noticeable side effects in SD rats at the above same dose for 9 days. The oral rat LD_{50} of As_2O_3 for investigation as mutagen, tumorigen and reproductive effector was known as 14.6 mg/kg[20].

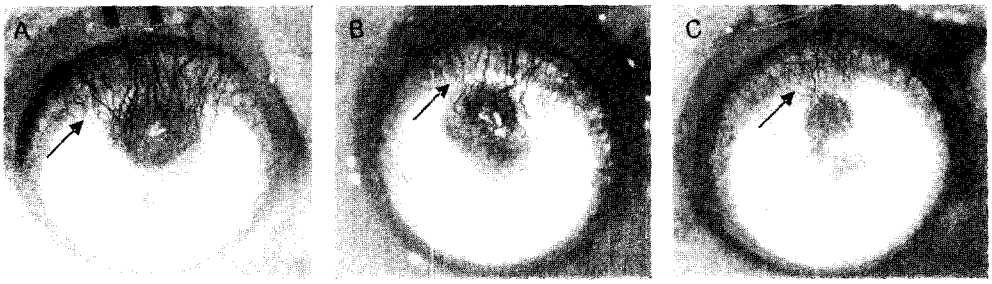

Fig 5. Appearance of angiogenesis on day 9 after VEGF pellet implantation on the rat corneal stroma and administration of arsenic compounds per oral. A. control group. B. As_2O_3 group. C. As_4O_6 group. °Ë: New vessels.

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*[8]. Angiogenic effect of VEGF in this study is in agreement with the Kenyon's research[7] who observed angiogenesis at the limbus of rat cornea 5 to 6 days after post-implantation using VEGF.

Lee *et al*[10] reported that VEGF had the dose-dependent relationship between VEGF-dose and matrix metalloproteinase (MMP) -2, 9 (gelatinase) in rat corea. Park *et al*[15] demonstrated that A_4O_6 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 A_4O_6[15]. In this study, As_4O_6 had better antiangiogenic effect than As_2O_3 in length, and area of vessels. As As_4O_6 inhibited the angiogenesis induced by VEGF, it was hypothesized that As_4O_6 might suppress autocrine secretion.

In recent years, arsenic trioxide (As_2O_3) has been used successfully in the treatment of patients with acute promyelocytic leukemia (APL). Some recent studies showed antivascular[11] and antiangiogenic activity of As_2O_3[17]. As_2O_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[22].

Roboz *et al*[17] reported that As_2O_3 inhibited capillary tubule and branch formation in an *in vitro* endothelial cell-differentiation assay. They believed that As_2O_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[13].

As_4O_6 significantly decreased the proliferation, migration, invasion, and tube formation of endothelial cells induced by the angiogenic factors bFGF. As_4O_6 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_4O_6 also had antiangiogenic effects on the new vessels induced by NGF in the rat cornea. However there are almost never antiangiogenic effecs in As_2O_3 group[23].

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[4].

It may be suggested that there were different mechanisms between As_4O_6 and As_2O_3 in antiangiogenic activity and As_4O_6 had more antiangiogenic effects than As_2O_3 in similar signaling pathways.

Conclusion

To evaluate the antiangiogenic effects of As_4O_6 and As_2O_3 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_4O_6 group were significantly less evident than those of control group and As_2O_3 group during experimental period ($P < 0.05$). In conclusion As_4O_6 had better antiangiogenic effects on the new vessel induced by VEGF in the rat cornea.

Acknowledgment

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랫드 각막에서 Vascular Endothelial Growth Factor(VEGF)로 유발시킨 신생혈관에 대한 As_4O_6의 혈관신생 억제효과

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요 약 : 본 연구는 VEGF로 유발된 랫드 각막 미세낭 모델에서 As_4O_6와 As_2O_3의 혈관신생 억제효과를 비교하기 위해 실시하였다. 20 ng VEGF가 함유된 펠렛을 이식하여 혈관신생을 유도하였다. 안과 검사후 정상적인 각막을 가진 Sprague-Dawley 랫드의 안구 60안을 이용하여 각 20안씩 무작위로 선정하여 대조군(비투약군), As_2O_3군(50mg/kg As_2O_3, 경구투여, 1일 1회), 그리고 As_4O_6군(50 mg/kg As_4O_6, 경구투여, 1일 1회)으로 나누어 실시하였다. 이식이 완료된 후, 신생혈관의 개수, 길이 및 신생혈관의 각도를 미세수술현미경하에서 이식 3일째부터 9일째까지 매일 검사하였다. 신생혈관의 넓이는 신생혈관의 길이와 각도를 수학적인 공식에 적용하여 계산하였다. 실험 기간동안 As_4O_6군의 신생혈관 개수, 길이, 각도 및 넓이가 대조군과 As_2O_3군에 비하여 유의성있게 억압되었다($P<0.05$). 이러한 결과 As_4O_6는 랫드 각막에서 VEGF에 의해 유도된 신생혈관에 As_2O_3보다 더 뛰어난 억제효과를 가지고 있음을 보여주었다.

주요어 : As_4O_6, As_2O_3, VEGF, 혈관신생억제, 랫드