FULL PAPER *Pharmacology*

Reverse Effects of Tetraarsenic Oxide on the Angiogenesis Induced by Nerve Growth Factor in the Rat Cornea

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ABSTRACT. To compare the antiangiogenic effects of tetraarsenic oxide (As_4O_6) with those of diarsenic oxide (As_2O_3) in the rat cornea, rat cornea micropocket assay was conducted to induce angiogenesis by implantation of the pellet contained 1.0 *n*g of nerve growth factor (NGF). Ten of thirty eyes of Sprague-Dawley rats were randomly assigned to one of three groups, namely, control group (no medication), As_2O_3 group (50 mg/kg As_2O_3, PO, s.i.d.), and As_4O_6 group (50 mg/kg As_4O_6, PO, s.i.d.). After implantation, the number of new vessels, vessel length and clock hour of neovascularization were examined under the microscope from day 3 to day 7. The area of neovascularization was calculated using a mathematical formula. Although new vessels in control and As_2O_3 groups were first noticed at day 3, whereas those of As_4O_6 group were first observed on day 5. The number, length, clock hour of neovascularization and areas of the vessels in As_4O_6 group showed more significant inhibition than those of control and As_2O_3 groups from day 5 ($P<0.05$). However, there were no differences in all parameters between control group and As_2O_3 group during the entire study period. These results showed that As_4O_6 had antiangiogenic effects on the new vessels induced by NGF in the rat cornea.

KEY WORDS: As_4O_6, As_2O_3, antiangiogenesis, NGF, rat.

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Arsenic is a natural material used as a medicine for more than 2,400 years. At one time, it was thought to be a panacea for all types of diseases, including chronic myelogenous leukemia (CML) and Hodgkin's disease [24]. The use of arsenic derivative substances had declined in the past 100 years, even in the mid-1990s. Because of cumulative toxic effects and carcinogenicity of arsenic derivative substances, it indicated only for the treatment of trypanosomiasis [3]. The reemerging of arsenic therapy occurred in the 1970s when physicians in China began using arsenic trioxide as part of a treatment of acute promyelocytic leukemia (APL). Many studies in human being were reported that arsenic preparations for APL patients had shown good prognosis [20, 22, 26]. The other studies about malignancies of the lung, bladder, skin [25], liver, and prostate [21] were also reported. Chronic exposure to arsenic causes a wide range of toxic effects and might lead to carcinoma by hypomethylation of DNA and deletion mutation [8, 25].

Although the mechanisms of arsenic trioxide in cancer therapy were not precisely defined, it was speculated that one of them was inhibition of angiogenesis [24]. Roboz [17] reported that arsenic trioxide inhibited the capillary tubule formation induced by vascular endothelial growth factor (VEGF). As_4O_6 is one of the arsenic compound derivatives, which was used as empirical anticancer agent in Korean alternative medicine. Recently, some studies had started to elucidate the mechanism of its anticancer effect, including apoptosis [13] and antiangiogenesis [14]. Park *et al.* [13] suggested that As_4O_6 might be a new arsenic compound which induced apoptosis in U937 leukemic cells at much lower concentration than As_2O_3. These two chemical have been proved different compounds with different physical and chemical characteristics, As_2O_3 has an infinite two dimensional sheet structure, whereas As_4O_6 has a discrete adamentyl structure [4].

NGF is also known to promote the neural differentiation and survival of several pheripheral and central neurons [1, 2]. Bonini *et al.* [5] reported therapeutic activity of topical administration of NGF in neurotrophic keratitis. Some studies reported that NGF had angiogenic effects associated with nerve growth effects in several nerve ganglions [12, 18, 19]. Seo *et al.* [19] reported the dose-dependent angiogenic effects of NGF on the rat cornea. In addition, Park *et al.* [14] reported that As_4O_6 inhibit angiogenic effect of basic fibroblast growth factor (bFGF) on the rat cornea. However, there have been no studies about antiangiogenic effects of As_4O_6 on the rat cornea neovascularization induced by NGF.

The aim of this study was to evaluate the reverse effects of As_4O_6 on neovascularization induced by NGF in the rat cornea.

MATERIALS AND METHODS

Experimental animals: Female and male Sprague—Dawley rats, weighing 250 to 350 g, were sacrificed in this study. The animals were allowed unrestricted access to pelleted food and tap water, and were confirmed to have no ophthalmic diseases by eye examination before pellet implanta-

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tion. All experimental procedures were carried out in accordance with the guide for the care and use of laboratory animals.

Experimental design: The entire groups were randomly divided into three groups; control group (no treatment group), As_2O_3 group (50 mg/kg As_2O_3, PO, s.i.d.), and As_4O_6 group (50 mg/kg A_4O_6, PO, s.i.d.). Each eye was a basic experiment unit.

Pellet preparation: Pellets were prepared according to the method by Polverini *et al.* [15]. Sterile casting solution was prepared by dissolving the poly-2-hydroxylethylmethacrylate (Hydron™, Sigma Co., U.S.A.) powder in absolute ethanol (12% w/v) with continuous stirring at 37°C for 24 hr. An equal volume of Hydron™ and sucralfate (Sigma Co., U.S.A.) were combined. Then 1.0 *n*g NGF was mixed with 2 μl of the above solution. This mixture was pipetted onto the surface of strerile teflon rods to make a pellet of 2 mm diameter. After dried at room temperature in a sterile environment for 1 to 2 hr, the pellets were stored at 4°C. Each pellet contained 1.0 *n*g of NGF according to the previously study [19].

Pellet implantation: Pellet implantation into rat corneas was conducted by the method previously described [15, 19]. 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., U.S.A.), and gently proptosed and secured by clamping the upper eyelid with a nontraumatic hemostat. Under a surgical microscope, 1.5 mm incision was made at the center of the cornea but not through it. An approximately 1.5 mm-wide curved microdissector was then inserted under the lip of the incision and gently blunt dissected through the stroma toward the limbus of the eye. Once the corneal pocket was made, the pellet was rehydrated with saline, and positioned down to the base of the pocket.

Corneas were examined daily with the aid of a surgical microscope to monitor angiogenic responses to NGF, and then antibiotic ointment (Terramycin™, Pfizer Co., Korea) without corticosteroids was applied to the eyes once a day.

Biomicroscopic examination: Eyes were examined under a surgical microscope (Leica M651™) daily from 3rd day after pellet implantation for 5 days. The number of vessels, length of vessels, and clock hour of neovascularization were saved with a digital camera in to the computer using a software (Image Tools, Ver. 2.0, The University of Texas Health Science Center in San Antonio, U.S.A.). Photographs of the rat cornea were made with a digital camera. Each photograph was analyzed at the same magnification with a computer program. The contiguous circumferential zone of neovascularization was measured as clock hours with a 360° reticule (where 30° of arc equalled 1 clock hour). The area of corneal neovascularization was determined with a reticule by measuring the vessel length (L) from the limbus and the number of clock hours (C) of limbus involved. Only the uniform contiguous band of neovascularization adjacent to the pellet was measured. The area of the circular band segment was calculated using following the formula: $C/12 \times 3.1416[r^2-(r-L)^2]$, C=clock hour (30°), r=2.5 mm, the measured radius of the rat cornea [7].

Statistical analysis: All numerical variables (number of vessels, length of vessels, clock hour of neovascularization and area of vessels) were assessed by use of repeated measure ANOVA with two factors; time, treatment (non medication, As_4O_6 or As_2O_3), and their interaction were examined. When significant difference was determined, multiple comparisons at each time points were made using Scheffe test. Values of $P<0.05$ were considered significant. All analysis were performed using the statistical package SAS 8.1 (SAS Institute, Cary NC).

RESULTS

The number of vessels: New vessels in As_4O_6 group were not detected until day 4. Limbic vessels of other groups began sprouting into the cornea on postoperative day 3. The number of vessels increased in all groups with time. The vessel number in As_4O_6 group was significantly fewer than control group and As_2O_3 group from day 5 to day 7 ($P<0.05$). However, there was no significant difference of vessel number in As_4O_6 group and control group during the observation period (Fig. 1).

The length of vessels: Vessel length changes in each group showed a similar pattern to the number of vessels. From day 5 to day 7 the vessel length in As_4O_6 group was significantly shorter than that of control and As_2O_3 groups ($P<0.05$). The vessel length in As_2O_3 group was somewhat longer than control group, but no difference was found (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 vessel number and length. There was no significant difference between control group and As_4O_6 group during the experimental

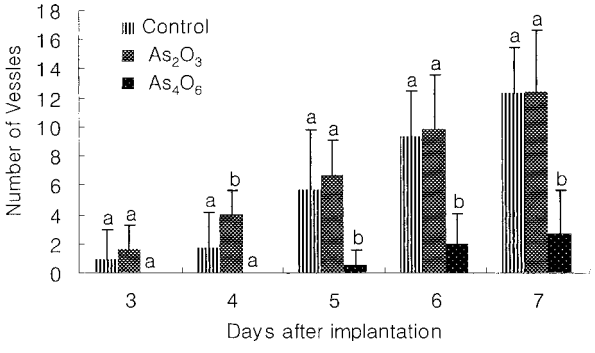

Fig. 1. Changes of the number of vessels after administration of arsenic compound daily in the rat cornea with NGF pellet implantation. The values are shown as mean ± SD. a, b: Different letters within same day mean significantly differences at $P<0.05$.

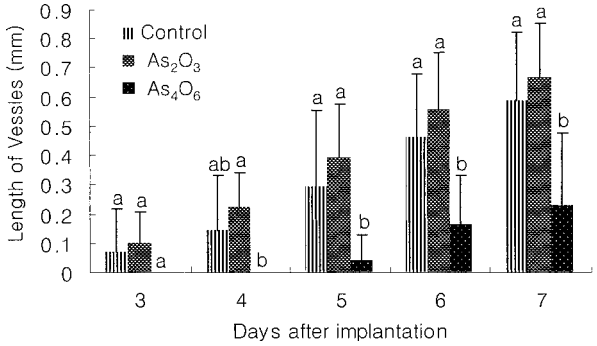

Fig. 2. Changes of the length of vessels after administration of arsenic compound daily on the rat cornea with NGF pellet implantation. The values are shown as mean ± SD. a, b: Different letters within same day mean significantly differences at $P<0.05$

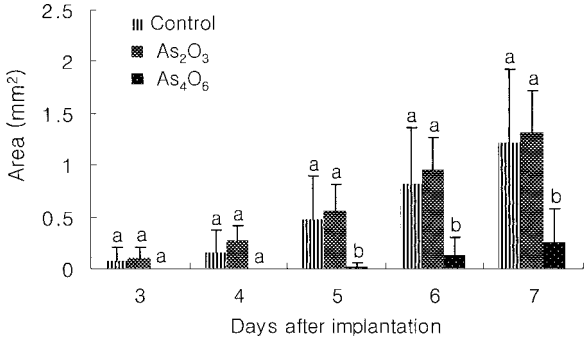

Fig. 4. Changes of area of neovascularization after administration of arsenic compound daily on the rat cornea with NGF pellet implantation. The values are shown as mean ± SD. a, b: Different letters within same day mean significantly differences at $P<0.05$.

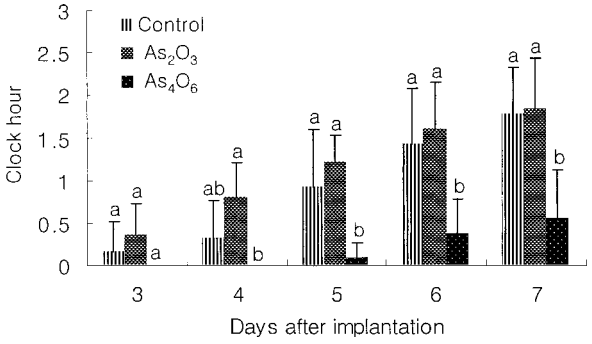

Fig. 3. Changes of the clock hour neovascularization after administration of arsenic compound daily on the rat cornea with NGF pellet implantation. The values are shown as mean ± SD. a, b: Different letters within same day mean significantly differences at $P<0.05$.

period. As_2O_3 group showed significantly narrower clock hours of neovascularization than in control group and As_2O_3 group from day 5 to day 7 ($P<0.05$; Fig. 3).

The area of vessels: As_4O_6 group was found to have significantly lesser vessel area than in control group and As_2O_3 group from day 5 to day 7. However, there was no significant difference in vessel areas between As_2O_3 group and control group ($P<0.05$; Figs. 4, 5).

DISCUSSION

This study showed that As_4O_6 inhibited angiogenesis induced by NGF in the rat cornea, whereas As_4O_6 did not have the antiangiogenic activity on NGF. It is one of the evidence that As_4O_6 is different physical and chemical compound from As_2O_3. Park *et al.* [14] also demonstrated that the oral administration of As_4O_6 at the dose of 50 mg/kg per day for 7 days to Sprague Dawley rats and for 22 days to C57BL/6 mice caused no noticeable side effects in contrast to the severe gastrointestinal toxicities of oral administration of As_2O_3 [20]. In this study, we also found As_4O_6 had no significant side effects in SD rats at the above same dose, whereas some rats given As_2O_3 had shown severe gastrointestinal problems. The oral rat LD_{50} of As_2O_3 is known as 14.6 mg/kg [23]. From the above study, As_4O_6 might be a much safer compound than As_2O_3. Park *et al.* [13] also demonstrated that As_4O_6 induced apoptosis in U937 leukemic cells at much lower concentration than As_2O_3. In our experiment, it was impossible to increase the dose of As_2O_3 because of the toxicities. Even though differences of absorbing efficiencies between these two arsenicals are not clarified yet, we might speculate from the results of our

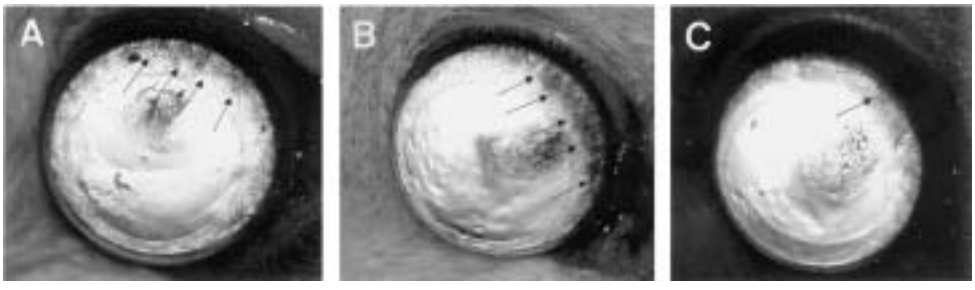

Fig. 5. Appearance of angiogenesis on day 7 after NGF pellet implantation on the rat corneal stroma and administration of arsenic compounds per oral. A. contorl group. B. As_2O_3 group. C. As_4O_6 group. ↑: New vessels.

experiments that two folds of As_4O_6 do not equal to the same amount of As_4O_6. In previous study [14], As_4O_6 inhibited the proliferation, migration into the denuded area and invasion through a layer of Matrigel of basic fibroblast growth factor (bFGF)—stimulated bovine capillary endothelial (BCE) cells in a dose-dependent manner in *in vitro* studies. To understanding the optimal dose of antiangiogenesis and LD_{50} of As_4O_6 in rats, further study should be needed.

NGF had been known to have angiogenic effect as well as neuroprotective and neurotrophic activity [1,2]. Recently, Cantarella *et al.* [6] had reported that human umbilical vein endothelial cell (HUVEC) proliferation triggered by NGF was specifically mediated by trk A via activation of the mitogen associated protein kinase (MAPK) pathway, and NGF also plays an autocrine role in endothelium for exerting angiogenic effect. As As_4O_6 inhibited the angiogenesis induced by NGF in this study, it was hypothesized that As_4O_6 suppressed autocrine secretion. Further reliable study should be followed to elucidate it.

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 [11]. The other known mechanism of As_2O_3 was apoptosis. Pu *et al.* [16] reported that As_2O_3 exerted its cytotoxic effect via the conventional apoptotic pathway involving reactive oxygen species (ROS) production, loss of mitochondrial membrane potential, activation of caspase-3 and internucleosomal DNA breakdown.

Different angiogenic inducers were likely to modulate the formation of new blood vessels via either similar or distinct signaling pathways. There were a total of 94 human genes with differential expression patterns in response to mitogen treatment. The expression patterns of thirty—two 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 meant that the mechanisms of two angiogenic inducers were different.

Judging from the different results of antiangiogenesis effect between As_2O_3 and As_4O_6 in this and previous studies [10], it was speculated that NGF and bFGF had different signaling pathways for the formation of new vessels and there were different mechanisms between As_4O_6 and As_2O_3 in antiangiogenic activity.

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