Comparisons of the Suppressive Effects on Tumor Growth by Different Combinations of Administration of Arsenic Trioxide and Hyperthermia

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Abstract: Arsenic trioxide (ATO), which is known to be an effective carcinostatic against acute promyelocytic leukemia (APL), has drawn attention throughout the world. Studies on the effectiveness of ATO against solid tumors have started, and progress is expected. In this study, we evaluate the differences in the suppressive effects on tumor growth between different combinations of ATO administration and hyperthermia.

Mice in which a squamous cell carcinoma (SCC-VII) had grown to 5-7 mm in the right femoral region were subjected to experiments in 5 groups. The groups consisted of the control group, hyperthermia alone group, ATO alone group, A-H group (ATO administration followed by 1-hour hyperthermia at an interval of 2 hours), and H-A group (1-hour hyperthermia followed by ATO administration at an interval of 2 hours). In each group, 10 mice were used for the measurement of the tumor volume, and 1 mouse for the pathological examination. The suppressive effects on tumor growth were examined in the A-H and H-A groups using changes in the tumor volume and pathological findings of the tumor.

The changes in the tumor volume demonstrated synergistic effects of combined hyperthermia and ATO administration, but there was no significant difference in the suppressive effects between the A-H and H-A groups. The histopathological examination demonstrated more histological changes in the
A-H and H-A groups than in the control, hyperthermia alone, and ATO alone groups, but there was no significant difference between the A-H and H-A groups. Our results indicate that there was no difference in the suppressive effects on tumor growth between the treatment sequences of ATO administration and hyperthermia at an interval of 2 hours.

Key Words: anti-tumor effect, arsenic trioxide, hyperthermia, solid tumor

Introduction

Arsenic was used as a drug in the fifteenth century. It was orally administered for the treatment of syphilis and trichophytosis in the sixteenth century, typhnia in the seventeenth century, and cancer in the nineteenth century. Simple arsenic is considered non-toxic, but arsenic compounds, such as hydride (AsH3), oxides (As2O3, H3AsO3), sulfide (As2S3), and halide (AsF3), are toxic. Chinese and American researchers have reported that arsenic trioxide (ATO, As2O3) induced apoptosis not only in recurrent acute promyelocytic leukemia (APL) but also in other leukemic cells, and alleviated leukemia. Administration of ATO for the treatment of APL was officially approved in June 2000 by the Food and Drug Administration (FDA) in the USA.

There have been a number of studies on the effects of ATO on patients with leukemia and leukemia-related cells, but only a few studies on the effects of ATO on solid tumors have been reported so far. Recently, basic studies using solid tumors, such as Signalerings Commissie Kanker (SCK) and frog skin angiotensin II (FSAII), are only recently begun. We found suppressive effects of ATO on growth of solid tumor using SCC-VII, and also found synergistic suppressive effects of hyperthermia after ATO administration on tumor growth.

In this study, we examined the suppressive effects of different treatment sequences of ATO administration and hyperthermia on tumor growth in C3H/Hej mice with SCC-VII by measuring the tumor size. Changes in tumor cells were examined by microscopic observation of necrosis in excised sections treated by Hematoxylin and Eosin staining (HE staining).

Materials and methods

Experimental animals

Male C3H/Hej mice were purchased from Nihon LSC Inc. In each of the control, hyperthermia alone, ATO alone, A-H, and H-A groups, 50 mice were used for the measurement of the tumor volume, and 5 mice for pathological examination (total, 55 mice). The weight of all the mice was 21-26 g, and the age was 6 weeks. The mice were fed at a temperature of 22±0.2°C and a humidity of 60±5% in lighting from 6:00 a.m. to 6:00 p.m. in an animal-feeding building. The mice were freely allowed to take solid CE-2 (Nihon Clea Inc.) and filtered tap water.

Tumor

SCC-VII tumor is a spontaneous cancer in C3H/Hej mice. SCC-VII cells stored at -80°C of a refrigerator were thawed and incubated in an incubator at a temperature of 37°C, a carbon dioxide concentration of 5%, and a sufficient humidity. 1×10⁶ SCC-VII cells/0.05 ml per mouse were
subcutaneously injected into the right femoral region using a 26G needle. The mice in which the tumor had grown to 7 mm in the right femoral region were subjected to experiments.

**Arsenic trioxide**

ATO powder was purchased from Nihon Wako Inc. The dose of ATO was 0.02 ml per 1 g of the body weight (8mg/kg).

**Hyperthermia**

Hyperthermia was achieved via water bath method, using warm water at a controlled temperature. Hyperthermia was performed by immersing the mouse into the water bath whose temperature was maintained at 42.5°C. Hyperthermia was prolonged for 1 hour, but the treatment duration at 42.5°C was 55 min because it took 5 min to elevate the temperature within the tumor to 42.5°C. The temperature in the constant-temperature water tank was continuously controlled by the thermo sensor (SBAC-40, SHIMADZU), and the error was ±0.2°C.

**Measurement of the tumor volume**

The mice whose tumor size in the right femoral region were bigger than 7 mm in diameter were

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**Fig. 1.** This figure shows the treatment methods in the control, hyperthermia alone, ATO alone, A-H, and H-A groups. The treatment was performed in each group shown with the arrow on the abscissa at the point shown with the arrow on the ordinate. No treatment was performed in the control group. In the hyperthermia alone group, hyperthermia (42.5°C) alone was prolonged for 1 hour, and in ATO alone group, ATO administration alone was performed. In the A-H group, ATO was administered, followed by 1-hour hyperthermia (42.5°C) at an interval of 2 hours, and in the H-A group, hyperthermia (42.5°C) was prolonged for 1 hour, followed by ATO administration at an interval of 2 hours. After the above treatment, the tumor diameter was measured every day. For histopathological examination, tumor lesions were removed 3 days after the treatment in all groups, and sections were examined.
divided into the control group, hyperthermia alone group, ATO alone group, A-H group (ATO
administration followed by 1-hour hyperthermia at an interval of 2 hours), and H-A group (1-hour
hyperthermia followed by ATO administration at an interval of 2 hours). Hyperthermia and ATO
administration were undertaken after measurement of the tumor diameter (Fig. 1). In each group, 10
mice were used for the measurement of the tumor volume. In the ATO administration groups, the dose
of 8 mg/kg ATO was equivalent to 0.02 ml per 1 g of the body weight (g). In the hyperthermia groups,
the right femoral region was immersed in water (42.5°C) for 1 hour. In the hyperthermia and ATO
administration groups, the interval between the two treatment methods was 2 hours.

The tumor diameter was measured every day with calipers, and the tumor volume was evaluated by
means of \((\pi/6)ab^2\) (a: major diameter of the tumor, b: minor diameter of the tumor). If the tumor
volume in the treatment groups was smaller than that in the control group, the tumor growth was
regarded as delayed. The suppressive effects of the treatment methods were evaluated by observing the
delay time of tumor growth. The period of tumor growth required for the attainment of the 4-fold larger
volume than the initial volume was determined, and the synergistic effects were evaluated in the A-H and
H-A groups.

Statistical analysis

The difference in the tumor volume between the A-H and H-A groups was examined by statistical
analysis. First, variances of the two groups were examined by the equal variance test (F test). Then,
if the variances were regarded as equal, the difference was examined by Student’s t-test, while if the
variances clearly differed, Welch’s t-test was used. A value of \(p < 0.05\) was regarded as significant.

The synergistic effects in the A-H and H-A groups were examined by determining the \(\omega\) value, which
is used for the evaluation of the combined effect type. The \(\omega\) value was determined using the equation
shown below. The combined effects were regarded as competitive when \(\omega < 1\), additive when \(\omega = 1\), and
synergistic when \(\omega > 1\). The period of tumor growth required for becoming four times bigger than the
initial volume was examined in all groups, and the delay of tumor growth was evaluated by comparing
the periods between the control group and the treatment groups. The suppressive effects of the
combination of ATO and hyperthermia were regarded as synergistic.

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\omega = \frac{\text{measurement value}}{\text{theoretical value}}
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Measurement value (days): Delay of the tumor growth in the A-H or H-A group
Theoretical value (days): Delay of the tumor growth in the ATO alone group + Delay of the tumor
growth in the hyperthermia alone group

Pathological examination

The tumor lesions were removed from the right femoral region of the mouse in each group 3 days
after the start of measurement of the tumor volume, and necrosis of the tumor in HE-stained sections was
examined by microscopy.
Results

Measurement of the tumor volume

Figure 2 shows the tumor volume. The ordinate shows the tumor volume (mm$^3$), and the abscissa the period (days) after the start of measurement. $	imes$ shows the control group, $\blacktriangle$ the hyperthermia (42.5°C) alone group, $\bigtriangleup$ the ATO alone group, $\blacklozenge$ the H-A group, and $\bigcirc$ the A-H group. Each point shows the mean $\pm$ SD.

Fig. 2. This figure shows the changes in the tumor volume determined using the tumor diameter measured after the treatment. The ordinate shows the tumor volume (mm$^3$), and the abscissa the period (days) after the start of measurement. $\times$ shows the control group, $\blacktriangle$ the hyperthermia (42.5°C) alone group, $\bigtriangleup$ the ATO alone group, $\blacklozenge$ the H-A group, and $\bigcirc$ the A-H group. Each point shows the mean $\pm$ SD.

As shown in Fig. 2, the tumor growth was most delayed in the H-A group, and almost the same delay was observed in the A-H group. The hyperthermia alone and ATO alone groups showed similar delay of the tumor growth. The F test of the difference in the tumor growth during the observation period between the A-H and H-A groups demonstrated that the variances were equal, and Student’s t-test demonstrated no difference in the tumor growth between the two groups. The period of tumor growth required for becoming four times bigger than the initial volume was 4.5 days in the control group, 6.0 days in the ATO alone group, 6.8 days in the hyperthermia alone group, 9.0 days in the H-A group, and 8.5 days in the A-H group. In other words, the delay of the tumor growth was 1.5 days in the ATO alone group, 2.3 days in the hyperthermia alone group, 4.5 days in the H-A group, and 4.0 days in the A-H group. When the delay of the tumor growth in the control group was regarded as 1, the ratio of the delay was 1.3 in the ATO alone group, 1.5 in the hyperthermia alone group, 2.0 in the H-A group, and 1.9 in the A-H group respectively. Based on these results, the $\omega$ value was calculated to be 1.2 in the H-A group and 1.1 in the A-H group, indicating that the suppressive effects were synergistic in both groups.
Histopathological examination

Fig. 3a to 3e are photomicrographs of HE-stained sections in the control, hyperthermia alone, ATO alone, H-A, and A-H groups, respectively. In the control group, tumor cells were homogeneously distributed, and the entire tumor lesion was well stained. In the hyperthermia alone group, the staining was slightly heterogeneous, and mild necrosis of the tumor cells and nuclear denaturation were observed.
In the ATO alone group, hemorrhage from some capillary blood vessels in the tumor lesion was detected, and slightly heterogeneous staining, mild necrosis of the tumor cells, and nuclear denaturation were observed, as in the hyperthermia alone group. In the H-A, and A-H groups, hemorrhage from capillary blood vessels in the tumor lesions was extensively observed, and the staining was heterogeneous. Furthermore, lack of cells and nuclear denaturation were extensively observed, and necrosis of the tumor cells were detected. However, there were no differences in the histological changes between the H-A and A-H groups.

Discussion

Our measurement of the tumor volume indicated that there was no difference in the suppressive effects on the tumor growth between the H-A and A-H groups, but the suppressive effects were high and synergistic in both groups. In other words, there was no difference in the suppressive effects on the tumor growth induced by hyperthermia and ATO administration at an interval of 2 hours irrespective of the treatment sequence, and the suppressive effects were synergistic. The histopathological examination demonstrated no difference in the histological changes in the tumor lesions between the A-H and H-A groups. Hemorrhage in wide areas of the tumor lesions and heterogeneous staining of the lesions were observed in the A-H and H-A groups compared to the control, hyperthermia alone, and ATO alone groups, indicating various histological changes in the combined treatment groups. These results indicated that there were more different histological changes in the combined treatment groups than in the single treatment groups, but the difference in the histological changes was not significant between the combined treatment groups.

The synergistic suppressive effects on the tumor growth in the A-H group may have been caused by enhancing the suppressive effects of hyperthermia by reducing of pH in the tumor lesions by ATO administration compared to the surrounding normal regions. ATO specifically attacks neogenetic blood vessels in tumor lesions, disrupts the blood flow, aggravates the nutritional condition in the tumor lesions, reduces oxygen supply, induces anaerobic metabolism, and accumulates lactic acid, leading to reduction of pH in the tumor lesions compared to the surrounding normal regions. Enhancing factors of suppression of tumor growth by hyperthermia are considered to be the cell cycle in the DNA-replicating phase, and low intracellular pH. The pH reduction in the tumor lesions by ATO administration occurred within the 2 hours before hyperthermia, and enhanced the suppression of tumor growth by hyperthermia, resulting in the synergistic effects. However, since this was caused by enhancement of the suppression of tumor growth by hyperthermia induced by the lower pH in the tumor lesions by ATO administration, there may be a different mechanism of the synergistic suppressive effects on tumor growth in the H-A group. Even though pH in the tumor lesions was lower than that in the surrounding normal regions by ATO administration after hyperthermia, the suppression of tumor growth by hyperthermia was not enhanced.

It has been reported that in vitro and in vivo administration of bleomycin, adriamycin, or nitromin with hyperthermia induced synergistic suppressive effects on tumor growth. As to such mechanisms, it has been suggested that hyperthermia caused functional changes in the cell membrane to increase its permeability to drugs allowing the entrance of drugs into the cells, to induce the binding of
epidermal growth factor (EGF), and to change the membrane potential leading to the channel opening \(^{13-16}\). Since the half-life of ATO in blood is reported to be about 12 hours \(^{17}\), the suppression of tumor growth by ATO was most likely sustained at the time of hyperthermia performed 2 hours after ATO administration, suggesting that functional changes in the cell membrane by hyperthermia induced the synergistic suppressive effects of ATO on the tumor growth. This mechanism may cause the synergistic suppressive effects on the tumor growth in the A-H group. The duration of functional changes in the cell membrane by hyperthermia is unknown, but possibly the changes were sustained at the time of ATO administration performed 2 hours after the completion of hyperthermia, and the synergistic suppressive effects of ATO on the tumor growth may have been observed in the H-A group. Therefore, this mechanism may induce the synergistic suppressive effects of ATO on the tumor growth not only in the A-H group but also in the H-A group.

**Conclusion**

As described above, the suppression of tumor growth by hyperthermia was enhanced by ATO administration, and the suppressive effects by ATO administration were enhanced by hyperthermia, suggesting that there was no significant difference in the suppressive effects on the tumor growth between the treatment sequences of hyperthermia and ATO administration at an interval of 2 hours. In addition to the above mechanism, there may be a complex combination of other factors, such as suppression by ATO administration of the recovery from potentially lethal damage (PLD) caused by hyperthermia and suppression by hyperthermia of the recovery from PLD caused by ATO administration.

The results of this study did not indicate whether higher suppressive effects on the SCC-VII growth were obtained by hyperthermia followed by ATO administration or ATO administration followed by hyperthermia at an interval of 2 hours.

**References**

三酸化二砒素と Hyperthermia の併用する
処理順番の違いによる腫瘍成長抑制効果の比較

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要旨：三酸化二砒素（以下 ATO）は急性前骨髄性白血病（APL）や他の白血病に対して効果的な抗癌剤であることが知られており、世界の注目を集めている。しかし、ATO の固形腫瘍に対する有効性は研究が始まって間もなく、今後の研究が期待されている。今回、我々は ATO 投与と Hyperthermia を併用する順番を変えたとき、腫瘍成長抑制効果に違いがあるかを検討した。腫瘍体積変化の結果から、併用処理による相乗効果が見られたが、両併用群間には腫瘍成長抑制効果に有意差は見られなかった。また、腫瘍病理標本上の結果からも大きな違いは見られなかった。