Induction of Apoptosis in Mice with B16 Melanoma by Treatment with the Antimicrotubule Agent TZT-1027

Rie Ikeda¹, Masanori Murakoshi¹, Masashi Ohtani¹, Masashi Tagawa¹,
Takashi Mikami¹, Tsugitaka Natsume², Junichi Watanabe²,
Motohiro Kobayashi² and Takaharu Nakayama¹

¹Safety Research Department, ²Pharmacological Research Department, Teikoku Hormone Mfg. Co. Ltd., 1604 Shimosakunobe, Takatsu-ku, Kawasaki 213-8522

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TZT-1027, an inhibitor of tubulin polymerization, is a derivative of dolastatin 10 isolated from the Indian Ocean sea hare *Dolabella auricularia*. TZT-1027 has good anticancer activity.

We investigated the effect on B16 melanoma of TZT-1027 in mice histopathologically and by agarose gel electrophoresis. At 1 mg/kg of TZT-1027, there was a good correlation between the number of mitosis-like cells and apoptotic bodies. In mitosis-like cells, the nuclear membrane had disappeared, and the chromosomes had morphological features typical of mitosis. However, not all cells in mitosis had proliferated.

In contrast, at 2 mg/kg of TZT-1027, many apoptotic bodies were detected soon after treatment, but these were not involved in the dynamic changes to mitosis-like cells. Furthermore, TUNEL-positive cells were clearly visible at 1 hr after treatment and the nuclei of these cells showed chromatin margination, cap-shape chromatin clamping, and nuclear condensation on electron microscopy. Therefore, we speculated that the effect on B16 melanoma cells of TZT-1027 differed with the treatment dose, and a dose of 2 mg/kg induces apoptosis in tumor cells by a direct mechanism.

Key words: TZT-1027, Antimicrotubule agent, B16 melanoma, Apoptosis, TUNEL method

I. Introduction

TZT-1027 is a derivative of dolastatin 10 isolated from the Indian Ocean sea hare *Dolabella auricularia* [8]. TZT-1027 is an antimicrotubule agent, and has strong antitumor activity compared with *vinca* alkaloids in several experimental mouse tumors [7]. We have used B16 melanomas in studies because TZT-1027 shows superior antitumor activity against them [7].

In a previous study [12], we investigated the effect on B16 melanoma of a single shot of TZT-1027 at 2 mg/kg in mice. We detected a ladder pattern of DNA bands (ladder pattern) characteristic of apoptosis within 3 hr of the treatment, and up until 48 hr. We confirmed that the number of apoptotic cells increased as the tumor was reduced by TZT-1027 treatment.

TZT-1027 inhibited microtubule polymerization, and arrested cells at the G2/M phase of the cycle [7, 9].

Therefore, we speculated that TZT-1027 induced cell cycle arrest and apoptosis during the tumor reduction process.

In the present study, we examined histopathologically and biochemically how TZT-1027 induces apoptosis in B16 melanoma. We attempted to observe the early changes in the tumor using the TUNEL method and ultrastructurally.

II. Materials and Methods

Animals and tumors

Female BDF1 mice were purchased from Charles River Japan Inc. (Kanagawa) at the age of six weeks. The animals were kept in a barrier-maintained room at $24 \pm 2^\circ\text{C}$ and $60 \pm 10\%$ relative humidity. The room was ventilated ten to eleven times per hr and provided with 12 hr of light (from 8:00 to 20:00). The animals were housed ten per poly-carbonated cage (CLEA Japan Inc., Tokyo) with solid food (MM-3, Funabashi Farm, Chiba) and tap water available *ad libitum*. One week was allowed for the animals to adjust to the laboratory conditions.

B16 melanomas were supplied by the Japanese Foun-

Correspondence to: Rie Ikeda, Safety Research Department, Teikoku Hormone Mfg. Co. Ltd., 1604 Shimosakunobe, Takatsu-ku, Kawasaki 213-8522, Japan.

dation for Cancer Research/Cancer Chemotherapy Center (Tokyo) and maintained by culture in C57BL/6 mice. BDF1 mice were subcutaneously transplanted with a 10% tumor cell suspension.

TZT-1027 is a structural modification of dolastatin 10 which was isolated from *Dolabella auricularia* [8]. TZT-1027 was dissolved in lactate buffer (pH 4-5) and administered at a final concentration of 0.5, 1 or 2 mg/kg by injection.

Experimental design

Dose dependency

The tumors that developed in twenty-seven BDF1 mice transplanted with B16 melanoma cells, served as material. Three animals were used as untreated controls (Group 1). In groups 2 to 4, twenty-four animals were given a single shot intravenously of TZT-1027 at 0.5, 1 and 2 mg/kg. The dose of 2 mg/kg was the one most toxic to the B16 melanoma cells and had no adverse effects on the animals [7]. Three (Group 1) or two (Groups 2 to 4) mice of each group were sacrificed at 0 (untreated control), 6, 9, 24 and 48 hr after the treatment, and their removed tumors were divided in two, and used for histopathological examination and agarose gel electrophoresis.

Early changes in B16 melanoma cells

Three of the animals described above served as the untreated control. Twelve animals were given a single shot intravenously of TZT-1027 2 mg/kg, and sacrificed at 0, 0.5, 1, 2 and 3 hr. Their tumors were divided in two; one was used for HE staining and TUNEL method, the other was used for analysis by TEM.

Histopathological examination

The tumors were fixed in 0.1 M phosphate buffered 10% formalin, embedded in paraffin, mounted and stained with hematoxylin and eosin (HE).

Detection of apoptosis

The 3'-OH ends of DNA fragments were visualized by using the Apop Tag detection kit (Intergen Co., NY). We used formalin-fixed and paraffin-embedded sections for this staining. After deparaffinization and hydration, the sections were incubated with 20 µg/mL proteinase K (Sigma Chemical Co., St. Louis) at room temperature for 15 min, washed with 0.01 M PBS, and then inactivated in absolute methanol containing 0.3% hydrogen peroxide. The sections were washed again with 0.01 M PBS, and once equilibration buffer had been applied, were incubated with terminal deoxynucleotidyl transferase in a reaction buffer containing digoxigenin dUTP at 37°C for 15 min. The reaction was stopped by placing the sections in a stop/wash buffer at 37°C for 30 min, and then anti-digoxigenin antibody coupled to peroxidase was applied for 30 min at room temperature. After being washed in 0.01 M PBS, the sections were treated in Graham-Karnovsky's reaction medium [4], which contained 0.02% 3, 3'-diaminobenzi-

dine (DAB, Wako Pure Chemical Industries, Osaka) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 5 to 10 min at room temperature. Finally, the sections were counterstained with 1% methyl green dissolved in veronal acetate buffer, pH 4.2.

Ultrastructural examination

Small pieces of tumor were fixed in 0.1 M phosphate-buffered 2.5% glutaraldehyde (pH 7.4) for 48 hr at 4°C under constant agitation. After being washed with 0.1 M phosphate-buffer, the tissues were post-fixed in 0.1 M phosphate-buffered 1% OsO₄ (pH 7.4) for 1.5 hr at 4°C. They were then dehydrated in a graded ethanol series and embedded in Quetol 812. Semi-ultrathin sections were cut by a Porter-Blum ultramicrotome, and were subjected to toluidine blue staining. After being trimmed, the ultrathin sections were prepared with a LKB ultra-microtome, double stained with uranyl acetate and lead citrate, and observed under a JEOL 1200EX electron microscope.

Detection of DNA fragmentation by agarose gel electrophoresis

The tumors were isolated, frozen in liquid nitrogen and stored at -80°C. DNA was extracted by conventional methods [1] with some modification. Frozen tumors were crushed into powder and treated with 50 µg/mL Proteinase K (Roche Diagnostics, Germany). The nucleotide solution was extracted with phenol/chloroform (1 : 1, vol/vol) incubated with 100 µg/mL RNase (Wako Pure Chemical Industries, Osaka). The DNA was extracted with phenol/chloroform again, and then precipitated in absolute ethanol. It was pelleted by centrifugation, washed with 70% ethanol, and dried. DNA samples thus obtained were resuspended in TE buffer (10 mM Tris-HCl buffer, pH 8.0, containing 1mM EDTA-Na), subjected to electrophoresis at 50V in 2% agarose gels and stained with 0.5 µg/mL ethidium bromide (Wako Pure Chemical Industries, Osaka) for 100 min. After electrophoresis, the DNA was visualized as bands on the gels by a UV transilluminator and the bands were photographed.

III. Results

Dose dependency

Ladder patterns obtained on agarose gel electrophoresis are shown in Fig. 1A-C. In group 1 (control), few mitosis-like cells and apoptotic bodies were detected in B16 melanoma (data not shown). Histopathological findings are shown in Fig. 2. In group 2 (0.5 mg/kg), the mitosis-like cells peaked in number at 24 hr after treatment, and were detected until 48 hr. The increase in number of apoptotic bodies was inconspicuous. In group 3 (1 mg/kg), the mitosis-like cells peaked in number at 9 hr after treatment, and were detected also until 48 hr. The number of apoptotic bodies was greatest at 24 hr after treatment. In group 4 (2 mg/kg), the number of

Fig. 1. DNA fragmentation of B16 melanoma treated intravenously with a single shot of TZT-1027 at 0.5 (A), 1 (B) and 2 (C) mg/kg. DNA was subjected to 2% agarose gel electrophoresis. B16 melanoma was isolated at 0, 6, 9, 24 and 48 hr after treatment. Two animals were used at each time point.

mitosis-like cells peaked at 9 hr after treatment. Many apoptotic bodies were already visible at 6 hr after treatment, and the number remained high until 48 hr. The results of agarose gel electrophoresis were consistent with the histopathological findings.

Early changes to B16 melanoma cells

In control B16 melanoma, few mitosis-like cells and apoptotic bodies were detected. On treatment with TZT-1027 at 2 mg/kg, the number of mitosis-like cells increased at 1 hr and the number of apoptotic bodies increased from 2 hr after treatment (Fig. 3A-C). In addition, the TUNEL-positive cell numbers clearly increased at 1 hr after treatment (Fig. 4A-C). The ultrastructural findings included the nuclear changes characteristic of apoptosis, such as chromatin margination, cap-shape chromatin clumping, nuclear condensation and so on (Fig. 5A, B). Nevertheless, no conspicuous changes to other organelles were observed.

IV. Discussion

We examined B16 melanoma treated intravenously with TZT-1027 at 0.5, 1 and 2 mg/kg histopathologically and by agarose gel electrophoresis.

In general, the antimicrotubule agent arrested cells at the G2/M phase of the cell cycle, and as a result, caused apoptosis [5, 6, 14]. TZT-1027 is toxic to tumor cells like other antimicrotubule agents [10, 16]. In the present study, at 1 mg/kg, maximum increases of apoptotic bodies were detected after the mitosis-like cells had accumulated.

At 2 mg/kg, however, the number of mitosis-like cells peaked at 9 hr after treatment. Many apoptotic bodies were already visible at 6 hr after treatment, and the number remained high until 48 hr after treatment. The correlation between the change in the number of mitosis-like cells and that of apoptotic bodies was not clear.

Furthermore, apoptotic bodies increased in number at 2 hr after treatment. The TUNEL-positive cell number apparently increased at 1 hr after treatment. The TUNEL method is based on the specific binding of terminal deoxynucleotidyl transferase to the 3'-OH ends of DNA fragments [2, 3]. In TUNEL-positive nuclei, DNA was also broken into fragments longer than the 180bp fragments of the laddering pattern. These longer fragments appeared before the laddering pattern. In addition, ultrastructural examination revealed nuclear changes characteristic of apoptosis. Therefore, our results indicated that at 2 mg/kg, TZT-1027 induced apoptosis early, but this was not responsible for the dynamic changes in mitosis-like cells.

The result is not explained simply by the arrest of cells at G2/M and the subsequent initiation of apoptosis. Shtil *et al.* [13] and Wang *et al.* [15] demonstrated that microtubule binding agents, taxol, taxotere, colchicine, vinblastine, vincristine, podophyllotoxin and nocodazole,

Fig. 2. The time-dependent changes in number of mitosis-like cells and apoptotic bodies in B16 melanoma after treatment intravenously with a single shot of TZT-1027. The data are the percentage of mitosis-like cells or apoptotic bodies among all tumor cells viewed at $\times 400$ magnification. Two animals were used at each time point. * The data could not be used for this examination because of insufficient development of tumors.

not only arrested the cell cycle and induced apoptosis, but also stimulated the activity of c-Jun N-terminal kinase 1 (JNK1) or Ras and apoptosis signal-regulating kinase 1 (ASK1). Their hypothesis is based on the following: Microtubules are highly labile cellular structures which can undergo rapid assembly and disassembly, and this dynamic behavior strongly influences microtubule function. Pharmacological or other modulations of the structure-function dynamics of microtubules may result in cellular stress [13, 15]. Interestingly, Shtil *et al.* [13] demonstrated that at low concentration, taxol and colchicine induced a delayed and modest change in microtubule structure and

did not activate JNK1 even after 24 hr.

In addition, Otani *et al.* [11] reported that in mice with colon 26, a solid cancer, hemorrhage was observed within 1 hr in the tumor cells treated with a single intravenous shot of 2 mg/kg TZT-1027. However, no major changes in hemorrhage were observed in normal tissues and organs. The tumor-selective hemorrhage occurred mainly in the peripheral area of the tumor mass. Measurements of tumoral hemoglobin content and dye permeation revealed that first the hemorrhage occurred and then the tumor blood flow stopped. In cultured human umbilical vein endothelial cells (HUVEC), TZT-1027 induced

Fig. 3. Light microscopic view of B16 melanoma treated intravenously with a single shot of TZT-1027 at 2 mg/kg. Although mitosis-like cells (arrow) appeared at 1 hr, apoptotic bodies (asterisk) were not detected until 2 hr after treatment. **A:** 1 hr after treatment. **B:** 2 hr after treatment. **C:** 6 hr after treatment. HE $\times 200$ (**A-C**).

Fig. 4. Light microscopic view of B16 melanoma labeled using the TUNEL method after treatment with a single shot of TZT-1027 at 2 mg/kg. The reaction-positive products (asterisks) were detected in the nuclei of cells from 1 hr after treatment. **A:** 1 hr after treatment. **B:** 2 hr after treatment. **C:** 6 hr after treatment. TUNEL method (Peroxidase-labeled antibody method) $\times 200$ (**A-C**).

Fig. 5. Electron microscopic view after 1 hr of B16 melanoma treated with a single shot of TZT-1027 at 2 mg/kg. The nuclear changes characteristic of apoptosis were observed, nevertheless, no conspicuous changes to other organelles were detected. Bar = 1 μ m, A; $\times$ 16,500, B; $\times$ 17,000.

marked cell contraction with membrane blebbing in 30 min. Therefore, it was strongly suggested that at 2 mg/kg, TZT-1027 selectivity affected the blood vessels of the tumor soon after treatment. The vascular damage was followed by continuous induction of apoptosis of the tumor cells, tumor tissue necrosis, and tumor regression.

In conclusion, we confirmed that the cellular toxic effects of TZT-1027 contributed to the induction of apoptosis in B16 melanoma. We concluded that TZT-1027 at 1 mg/kg induced apoptosis by arresting cells in the G2/M phase of the cycle, because there was a good correlation between the increases in mitosis-like cells and apoptotic bodies. In contrast, at the dose of 2 mg/kg, apoptotic bodies were detected soon after treatment, and were not involved in the dynamic changes to mitosis-like cells. Therefore, we speculated that the effect on B16 melanoma cells of TZT-1027 differed with the treatment dose, and a dose of 2 mg/kg induces apoptosis in tumor cells directly.

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VI. References

1. Compton, M. M. and Cidlowsky, J. A.: Rapid in vivo effects of glucocorticoid on the integrity of rat lymphocyte genomic deoxyribonucleic acid. *Endocrinology* 118; 38–45, 1986.
2. Gavrieli, Y., Sherman, Y. and Ben-Sasson, S. A.: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell Biol.* 119; 493–501, 1992.
3. Gorczyca, W., Gong, J. and Darzynkiewicz, Z.: Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 53; 1945–1951, 1993.
4. Graham, R. C. and Karnovsky, M. J.: The early stage of absorption of infected horseradish peroxidase in the proximal tubules of mouse kidney. *J. Histochem. Cytochem.* 14; 291–302, 1966.
5. Huschtscha, L. I., Bartier, W. A., Ross, C. E. A. and Tattersall, M. H. N.: Characteristics of cancer cell death after exposure to cytotoxic drugs in vitro. *Br. J. Cancer* 73; 54–60, 1996.
6. Kawamura, K.-I., Grabowski, D., Weizer, K., Bukowski, R. and Ganapathi, R.: Modulation of vinblastine cytotoxicity by dilantin (phenytoin) or the protein phosphatase inhibitor okadaic acid involves the potentiation of anti-mitotic effects and induction of apoptosis in human tumor cells. *Br. J. Cancer* 73; 183–188, 1996.
7. Kobayashi, M., Natsume, T., Tamaoki, S., Watanabe, J., Asano, H., Mikami, T., Miyazaki, K., Gondo, M., Sakakibara, K. and Tsukagoshi, S.: Antitumor activity of TZT-1027, a novel dolastatin 10 derivative. *Jpn. J. Cancer Res.* 88; 316–327, 1997.
8. Miyazaki, K., Kobayashi, M., Natsume, T., Gondo, M.,

- Mikami, T., Sakakibara, K. and Tsukagoshi, S.: Synthesis and antitumor activity of novel dolastatin 10 analogs. *Pharm. Bull.* 43; 1706-1718, 1995.
9. Mohammad, R. M., Pettit, G. R., Almatchy, V. P., Wall, N., Varterasian, M. and Al-Katib, A.: Synergistic interaction of selected marine animal anticancer drugs against human diffuse large cell lymphoma. *Anti-Cancer Drugs* 9; 149-156, 1998.
 10. Mohammad, R. M., Limvarapuss, C., Wall, N. R., Hamdy, N., Beck, F. W. J., Pettit, G. R. and Al-Katib, A.: A new tubulin polymerization inhibitor, auristatin PE, induces tumor regression in a human Waldenstrom's macroglobulinemia xenograft model. *Int. J. Oncol.* 15; 367-372, 1999.
 11. Otani, M., Murakoshi, M., Isobe, M., Mikami, T., Natsume, T., Kobayashi, M. and Nakayama, T.: An antimicrotubule agent TZT-1027 disrupts tumor vasculature and induces apoptosis. *Jpn. J. Cancer Res.* 91; 837-844, 2000.
 12. Otani, M., Isobe, M., Usui, T., Suzuki, M., Murakoshi, M., Kobayashi, M., Watanabe, J., Mikami, T. and Tsukagoshi, S.: Induction of apoptosis by TZT-1027, a novel derivative of dolastatin 10, in murine solid tumor in vivo. Proceeding of the Japanese Cancer Association (2188 I am M), 54th Annual Meeting, October, 1995.
 13. Shtil, A. A., Mandlekar, S., Yu, R., Walter, R. J., Hagen, K., Tan, T-H., Roninson, I. B. and Kong, A-N., T.: Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* 18; 377-384, 1999.
 14. Wahl, A. F., Donaldson, K. L., Fairchild, C., Lee, F. Y. F., Foster, S. A., Demers, G. W. and Galloway, D. A.: Loss of normal p53 function confers sensitization to Taxol by increasing G2/M arrest and apoptosis. *Nature Med.* 2; 72-79, 1996.
 15. Wang, T-H., Wang, H-S., Ichijo, H., Giannakakou, P., Foster, J. S., Fojo, T. and Wimalasena, J.: Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. *J. Biol. Chem.* 273; 4928-4936, 1998.
 16. Watanabe, J., Natsume, T., Fujio, N., Miyasaka, K. and Kobayashi, M.: Induction of apoptosis in human cancer cells by TZT-1027, an antimicrotubule agent. *Apoptosis* 5; 2000 (in press).

Fig. 1. Endothelial cell sprouts seen in well differentiated endometrioid adenocarcinoma. (CD34 by LSAB method, $\times 100$, Scale bar = 10 μm)

Fig. 2. Finger-like processes seen in well differentiated endometrioid adenocarcinoma. (CD34 by LSAB method, $\times 100$, Scale bar = 10 μm)

Fig. 3. Cul-de-sacs seen in well differentiated endometrioid adenocarcinoma. (CD34 and SMA by LSAB method, $\times 40$, Scale bar = 10 μm)

vessels. Immunohistochemical staining was performed according to the cell type using the labeled streptavidin biotin (LSAB) method with a monoclonal antibody to CD34 (Nichirei, Tokyo, Japan), an endothelial cell marker, as the primary antibody. Coloration was achieved using 3,3'-diaminobenzidine (DAB) to which 0.02% by volume of 0.005% hydrogen peroxide had been added. Staining was evaluated by comparison to a negative control. Because cellular overlapping in endometrial specimens is intense and tends to cause physical negativity, all of the reactions and washings were performed in shakers usually used for tissue fixation in a pathological laboratory to break up uneven antibody reactions.

Since new vessels have the structure of blood capillaries, in this study we only targeted CD34-positive blood capillaries composed of 1-2 layers of endothelial cells. To exclude artifacts, only vessels with smooth, round tips were targeted. The morphology of the vessels was examined through the 40-power objective of a light microscope.

The blood capillaries were composed of endothelial cells surrounded by pericytes. However, some of the CD34-positive blood capillaries contained cells that could not be morphologically confirmed as pericytes. To confirm whether pericytes were present in such blood capillaries, the antibodies were detached using a 0.1 M glycine HCl buffer (pH 2.2) and re-labelled using an alkaline phosphatase (ALP)-labeling LSAB method, with α -smooth muscle actin (SMA; DAKO, Kyoto, Japan) as the marker. The specimens were then stained red with fast red violet LB salt (Sigma, Tokyo, Japan). Levamisole (DAKO, Kyoto, Japan) and L-phenylalanine (Sigma, Tokyo, Japan) were used to eliminate endogenous ALP activity.

In addition, the immunoperoxidase technique using the monoclonal antibody Ki-67 (MIB-1) produced by IMMUNOTECH (Marseille, France) was applied to the other preparations fixed with 95% ethanol to certify the ability of the endothelial cells to undergo cell proliferation.

III. Results

Blood capillaries are usually anastomosed with each other and distributed throughout tissues in a network pattern. Many blood capillaries exhibiting a network pattern were noted in this study, but microprojections on the sides of the blood capillaries forming networks were also seen (Fig. 1). These projections were CD34-positive and continuous with the endothelial cells that composed the vascular network.

Finger-like processes that were slightly larger than the microprojections were also observed in a portion of the vascular network (Fig. 2). The ends of these processes were smooth and round, and one to several deformed nuclei were sometimes observed.

Blood capillaries whose ends were closed (cul-de-sacs) and that extended into avascular areas at the margin (avascular fields) were also seen in the network (Fig. 3).