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Therapeutic and imaging capacity of tumor-localizing radiosensitive Mn-porphyrin on SCCVII tumor-bearing C3H/He mice

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We synthesized a radiosensitizer KADTF, consisting of a hypoxic radiosensitizer, KU2280, bound to the side chain group of Mn-metalloporphyrin, which accumulates in tumor tissue. In the *in vitro* colony-forming activity test using HeLa cells, KADTF enhanced the effect of radiation under hypoxic conditions. Radiation therapy at 20 Gy, 1.5 h after infusion of KADTF (0.15 mM/kg), inhibited tumor growth more markedly than did a single radiation treatment. A clear tumor MR images of the SCCVII tumor was obtained at 1.5 h after administration of KADTF (0.1 mM/kg).

Key words: Hypoxic radiosensitizer, imaging agent, low neuro-toxicity, Mn-porphyrin, tumor localizing agent.

Introduction

Although many drugs have been found to enhance the radiosensitivity of hypoxic cells *in vitro* and some of them exhibited marked therapeutic effects in animal tumor models, clinical application has often failed because of insufficient uptake of drugs in solid tumors and neurotoxicity.[1–3] Thus, the development of radiosensitizers with higher tumor affinity and less toxicity is essential for achieving a high sensitizing enhancement ratio (SER) in clinical use. Porphyrin derivatives accumulate in malignant tumor tissue. Recently, various investigators developed diagnostic or therapeutic drugs for cancer using porphyrin derivatives.[4,5]

To date, we have synthesized approximately 950 kinds of porphyrin derivatives, studied their side chain structures and affinities for tumor tissues, and the mechanism of their physico-chemical action, and reported the possibilities of using porphyrins as a new method of cancer diagnosis and treatment.[6–10]

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This short article is our first report on the development of a hypoxic radiosensitizer which accumulates in tumor tissues and produces clear tumor MR images.

Materials and methods

Chemicals

KU2285, an excellent hypoxic radiosensitizer, was selected from the series of compounds of KU reported by Shibamoto *et al.* and a precursor of KU2285, KU2280, was adopted as side chain group for the convenience of synthesis.[11–14] We synthesized KADTF which possesses KU2280 in a structure fundamentally similar to that of STA-R12, a Mn-metalloporphyrin that accumulated well in tumor tissue as a tumor-positive scintigraphic agent.[15,16] KADTF was expected to be useful as a tumor imaging agent for MRI, because Mn-porphyrin is a high T_1 time suppressive substance. The chemical structures of these agents are shown in Figure 1.

Tumors

The tumor cell line SCCVII, a squamous cell carcinoma implanted in C3H/He mice, was kindly supplied by Dr U Shibamoto, Kyoto University.[13] The cell line was maintained by alternate passage *in vitro* and *in vivo* in syngeneic mice or stocked in a deep freeze. The tumor cells were maintained *in vitro* in Eagle's minimum essential medium (MEM) supplemented with 12.5% fetal bovine serum. All tumor cells were collected from a monolayer culture and approximately 1.0×10^5 cells were inoculated s.c. in the right thigh of syngeneic female mice aged 8–11

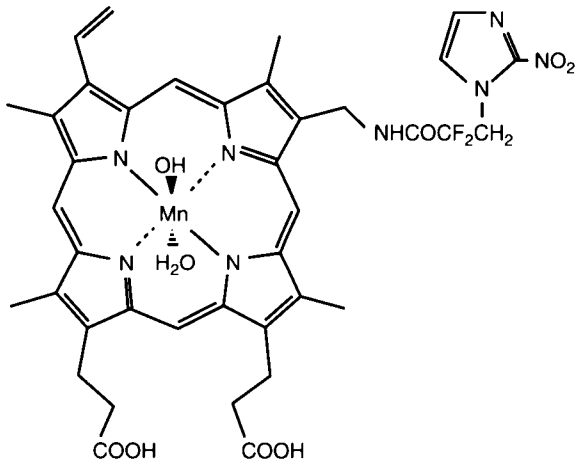

Figure 1. Chemical structure of KADTF. Molecular weight: 855.9.

weeks. The s.c. tumors reached 500–700 mm^3 in volume after 14–16 days and were used for experiments.

Assay for cell survival (*in vitro* colony formation assay)

HeLa cells adjusted to 10^5 were scattered in plastic Petri dishes (Corning; 6 cm in diameter) and pre-incubated for 3–4 days. The medium in each dish was exchanged for new medium to which various drugs (0.5, 1.0 and 1.5 mM) were added before a 2 h incubation. To produce the anoxic conditions, dishes containing the above drugs were placed in plastic chambers (modular incubator chamber/Bill-ups-Rothenberg, CA) and the air in them aspirated. Then, 95% N_2 + 5% CO_2 gas was insufflated. After this procedure was repeated three times, the valve cock was fastened and 40 min later irradiation (0, 10 and 16 Gy) was performed using an MBR1520 type X-ray irradiation system (Hitachi Medico, Tokyo, Japan; 150 kV, 15 mA, 0.5 Al + 0.5 Cu). After irradiation, the cells were separated from each dish (0.02% trypsin and 0.05% EDTA) and the cell count adjusted to 10^5. Dilution series were prepared and cells were scattered at various cell counts on 5 cm Petri dishes for 10–14 days of incubation. After colony formation, the medium was removed and the cells were fixed in formalin after being washed in phosphate buffer solution (PBS). Methylene blue staining was performed. Cells with the optimum numbers of colonies (50–100) were selected in each group and the number of cells was counted, followed by calculation of surviving fractions.

In vivo biodistribution study of KADTF

KADTF was dissolved in Tris–NaCl buffer and injected into the tail vein of SCCVII tumor-bearing C3H/He mice. The animals were then placed in metabolic cages. Three animals were killed at each of the five time points (pre-treatment, 30 min, 90 min, 3 h and 6 h after i.v. injection). After various tissues were removed they were weighed and dissolved in HON solution. The solutions were filtered through a 0.1 μm filter (Basseless, Dassel, Germany) and the metal concentration determined using an inductively coupled plasma atomic emission spectrometer (ICP-AES; Fissile Instruments, Mainz-Castle, Germany). Branch values (Mn background) were measured in untreated control animals.

Growth delay assay

KADTF was i.v. injected at a concentration of 0.15 mM for evaluating the *in vivo* effects and 1.5 h later the mice were irradiated (20 Gy) using a Mitsubishi LINEAC ML M2B radiation system under pentobarbital anesthesia (44 mM/kg). The accelerating effect of KADTF on radiation was evaluated from the tumor growth suppression rate.

In vivo NMR imaging

MRI was carried out on a Siemens Magnetron H15 (63 SP; Siemens, Berlin, Germany) operating at 63 MHz (1.5 T). For imaging animals, a circularity polarized head coil, 27 cm in diameter, was used as a r.f. receiver, with the clinical imaging body coil (55 cm in diameter) used as a transmitter. Animals were anesthetized with pentobarbital and taped prone onto a styrene foam plate placed in the CP head coil. T_1 relaxation times were obtained from *in vivo* imaging experiments using a T_1 enhanced sequence package provided by the manufacturer (T_1 weighted image). The image intensities averaged over three measurements calculated by drawing a region of interest (ROI) on the tumor, normal muscle in the contra lateral hind limb, lungs, brain, liver and kidneys were recorded in each set of images before and at various time intervals after administration of KADTF (0.1 mM/kg) for evaluation. The relative change in the average image intensity and image intensity normalized with the standard at various time intervals over the pre-injection value provided information regarding the relative concentration and transit of injected KADTF.

Results and discussion

As shown in Figure 2, KADTF showed no radiosensitivity enhancement effect under normoxic conditions, but had radiosensitivity enhancement effects under hypoxic conditions in a dose-dependent manner. It was confirmed that the effects of 2-imidazole residue remained even after it bound to porphyrin. The amount of Mn, which is present in the central structure of KADTF, was measured by the ICP method and used to estimate the distribution of i.v. injected KADTF in mouse organs including tumors. As shown in Figure 3, KADTF increased in the tumor tissues to reach a maximum 90 min after i.v. injection and then gradually decreased. The level of KADTF was higher in the liver and kidneys, both of which are excretory organs, than in tumors. Particularly, it was low in muscles and the brain, and the ratios of the KADTF level in tumors/muscles and tumors/brain were 6.7 and 7.5 at 90 min after injection, respectively, indicating its accumulation in tumor tissues and a decrease in its affinity in the nervous system caused by porphyrin. Figure 4 shows T_1-weighted images of the C3H/He mouse transplanted with the SCCVII tumor after i.v. injection of KADTF obtained by MRI. Immediately after i.v. injection, the liver and kidneys were strongly imaged, and after 30 min the contrast of tumors started to become clear. After 90 min the internal structures of tumor tissue, which had not been seen before administration, became clear. A rapid decrease in the level of KADTF in organs other than tumors, such as

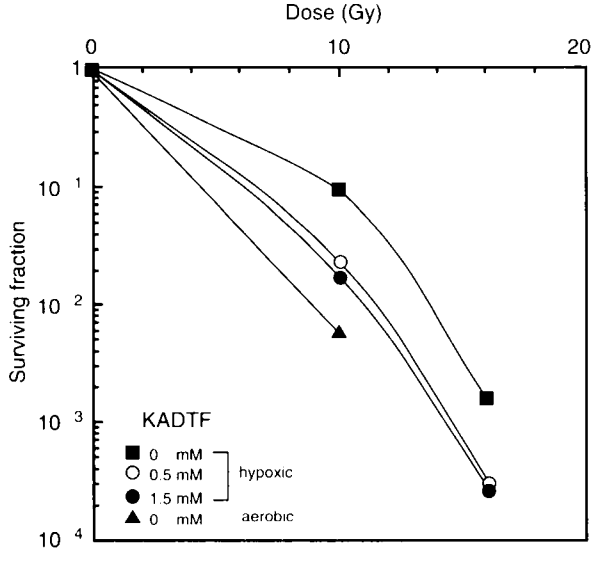

Figure 2. Survival curves for irradiated Hela cells under normoxic and hypoxic condition. Vertical axis: surviving fraction. Horizontal axis: radiation dose.

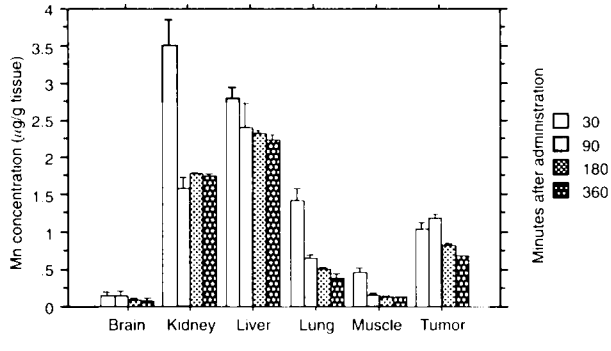

Figure 3. Mn concentration (μg/g tissue) of various tissues including tumor after administration of KADTF (0.1 mM/kg).

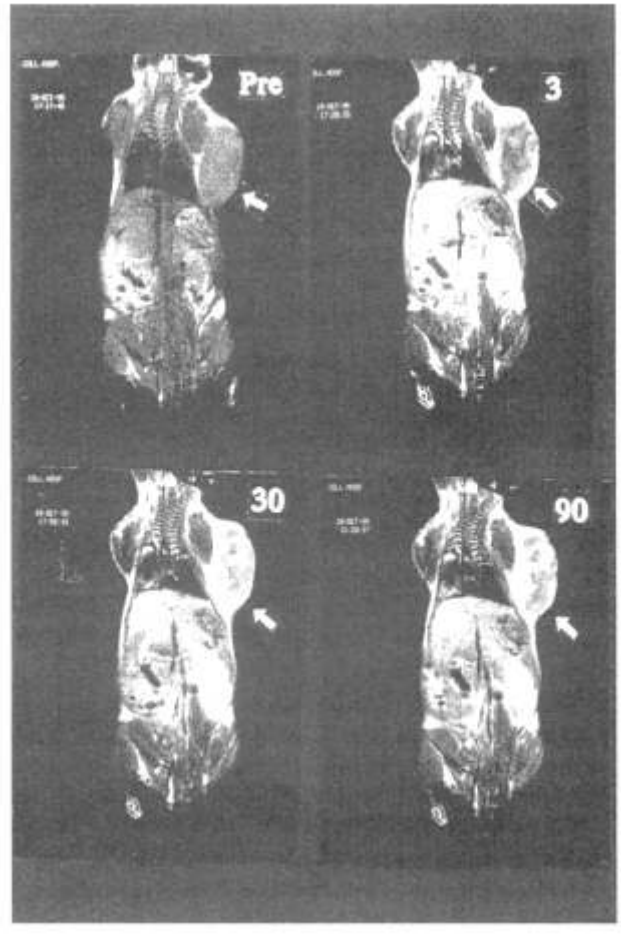

Figure 4. Coronal MR T_1-weighted images of SCCVII tumor-bearing C3H/He mouse after i.v. injection of KADTF (0.1 mM/kg). The figure at the right shoulder of each image indicates minutes after administration. The arrow indicates the tumor.

the lung, kidneys and muscles, was observed in the images. Figure 5 shows the distribution of KADTF estimated by changes in signals after administration obtained by setting ROI in T_1-weighted images by MRI. At 90 min after administration, the rate of change of the KADTF distribution was the largest in tumors, suggesting its accumulation in tumors. Figure 6 shows the effect of LINEAC irradiation (20 Gy) expressed as the mean ratio of the relative tumor volume in SCCVII tumor-bearing C3H/He mice (seven mice per group) which had been i.v. injected with KADTF (0.15 mM/kg). A single radiation therapy (20 Gy) was significantly different from the non-treated group ($p < 0.05$ by t-test) in the rate of change of the tumor volume. The effect of KADTF injection plus 20 Gy radiation therapy was also more significantly pronounced than single radiation therapy of the same dose ($p < 0.01$ by t-test).

A number of hypoxic radiosensitizers have been developed and some of them have been clinically used; however, sufficient therapeutic effects have not been obtained due to side effects such as neuropathy. Shibamoto *et al.* developed fluorinated 2-nitroimidazoles and demonstrated their good radiosensitivity enhancement effect.[11] We focused on one of the derivatives, KU2285, and synthesized KADTF, a compound consisting of a precursor of KU2285 and Mn-porphyrin. The precursor was used for reasons of synthetic reactions. The molecular weight of KU2280 is 235.2, comprising only one-third of that of KADTF (855.9). A dose of 0.15 mM/kg KADTF is equivalent to 35.3 mM/kg KU2280, which is not sufficient to obtain radiotherapeutic effects.[14] On the other hand, KADTF had significant therapeutic effects. These results suggested that Mn-porphyrin promoted the transfer of the 2-nitroimidazole residue to tumor tissues. Although examinations in the peripheral nervous system were not performed in this study, there was almost no accumulation of KADTF in the brain, suggesting that the use of KADTF reduces side effects such as central neuropathy. The LD_{50} of KADTF in C3H mice was 0.8 mM/kg or higher (unpublished data). KADTF with Mn in its backbone can markedly reduce T_1 in MRI. The molar relaxivity $1/T_1$ (mMS)$^{-1}$ of Gd–DTPA, which is used as a contrast medium for MRI, was 5.05 at 37°C in water at 10.7 Hz, while that of KADTF under the same conditions was 7.15. This indicates that KADTF has better T_1 reduction at the same mole fraction and its usefulness for a MRI contrast medium.

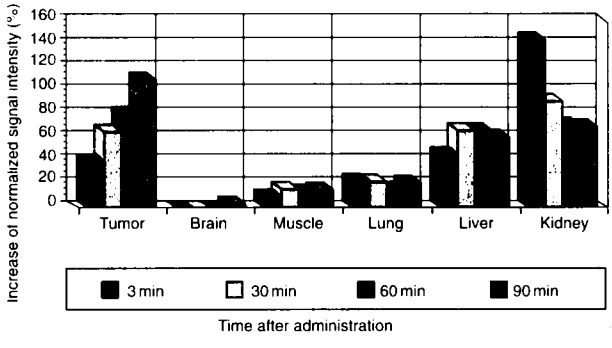

Figure 5. Percent increase of normalized signal intensity in MRI T_1 weighted images of various tissues.

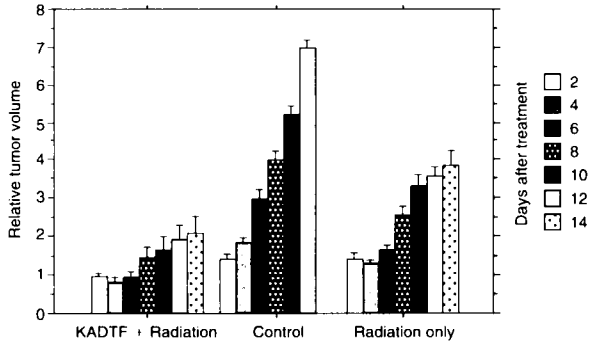

Figure 6. Changes of mean ratio of the relative tumor volume in SCCVII tumor-bearing C3H/He mouse after treatment (control, radiation only and KADTF plus radiation). Bar = average ± SD.

A number of possibilities have been suggested as to the accumulation mechanism of porphyrin in tumors. We speculate that porphyrin is taken up in cancer cells by activated endocytosis, based on the affinity of porphyrin for LDL and transferrin.[15] We have demonstrated the good imaging ability of tumor scintigraphic media containing Mn-porphyrin such as [^{99m}Tc]ATN-10 or [^{99m}Tc]STA-R12, the latter of which contains the same basic structure as KADTF.[16] Since the triplet state of Mn-porphyrin is shorter than milliseconds due to the characteristics of Mn, activated oxygen is hardly produced by photochemical reactions, causing no side effects such as phototoxicity.[17] As we have shown previously, replacement of the central metal atom in some porphyrins with Mn did not alter their ability to accumulate in tumor tissues.[8] In addition, since Mn-porphyrins have the ability to shorten T_1 in MRI, they are expected to be derivatives for good carriers of diagnostic and therapeutic drugs for tumors.[18] The results of this study show the possibility of effective radiotherapy by MRI using KADTF as well as its ability in detecting tumors. Further studies are necessary for its clinical use.

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