JRR-4 facility for animal irradiation experiments

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Abstract

A small animal irradiation facility is critical for providing optimal radiation dose distributions for pre-clinical studies. Animal neutron irradiation experiments cause many problems to solve, as the achievements of the permissions from the animal care office and using committee. At the same time, it is necessary to make radioprotection controls and to take care of radiation waste management. Animal irradiation was possible only few days per month because of shared nuclear reactor. As JRR-4 has no beam shutter, exclusive use of reactor was needed. To avoid this time consuming procedure, we constructed a simple facility for animal irradiation at JRR-4.
This system consists of some animal holders, a manual loader and some visual monitors. Animal holders can be filled with anesthetic gas, so that the mice are under general anesthesia during the irradiation. Rearing facilities were constructed into the radiation controlled area. Tumor control test by boron agents and neutron irradiation were put into practice. Unfortunately, a problem at the reactor caused a neutron outage which will last for about 3 years. This paper focuses on simulated neutron distribution and simulated absorbed dose in the animal body.

Keywords: BNCT, JAEA, JRR-4, animal irradiation

1. Introduction

The small animal irradiation facility at the Institute for Reactor Research should be able to ethically treat animals, and should have the proper equipment, personnel and problem-solving capabilities, necessary for such kind of studies. The management of such a facility requires much more than the routine control of the radioactive materials and the radioprotection of the users and animals.

Despite the complexities and expense of running such a facility, experiments evaluating the effects of radiation alone or in combination with other agents on tumor proliferation in small animals are indispensable for drug development and for the improvement of irradiation techniques.

The following accomplishments using the JAEA (Japan Atomic Energy Agency) research reactor JRR-4 (JAEA Research Reactor-4) were so far achieved.
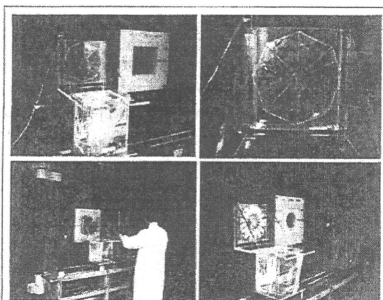
The reactor was available only few days during the year, we examined current situations, obstacles and issues involved in the development of the small animal irradiation experiments in the JRR-4 furnace, and reported on the neutron beam distribution and the simulation of the dose absorbed by the animal body.

2. Materials and Methods

The irradiation system consists of animal holders, a manual loader and visual monitors. Animal holders can be filled with the anesthetic gas, so that mice can be irradiated under anesthesia, allowing for a safer and a more accurate dose administration. Breeding facilities were constructed into the radiation-controlled area. Tumor size control and neutron irradiation tests using boron agents were established and safely carried out.

Using the newly designed animal irradiation devices, 4-6 animals can be irradiated at a time using the rails method, which is the newly designed animal irradiation devices as figure a. In addition, it also allows for a more uniform irradiation of the animals (Figure a).
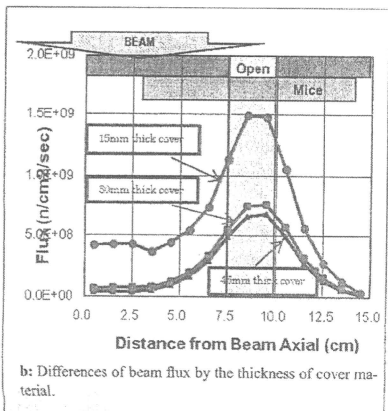
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a: The newly designed animal irradiation devices.

Even if the beam is stopped directly by the shield, because of the characteristic of the neutron flux, it can be deflected and reach other non-covered area, parts of the body, such as the head and the legs. So we must develop the new device to avoid this problem in the future.Because of the flux in the peripheral zone of the 20 cm beam was largely depressed, the flux in the peripheral zone was the same as that from the 15 cm beam. Therefore, no major difference was observed between the 20 cm and 15 cm beams in this evaluation (Figure c).

c: Differences of beam flux distribution by 15 cm and 20 cm collimator diameter with cover material.

Using our system with the JCDS (JAEA Computational Dosimetry System) that Kumada (2003) developed for dose calculation, the location for animal irradiation was reproduced and the dose was simulated. To determine the partial irradiation, we tested the irradiation setup most suitable to treat that tumor models. And we also examined the influence of an acrylic cover, boron rubber, and the LiF cover belt.
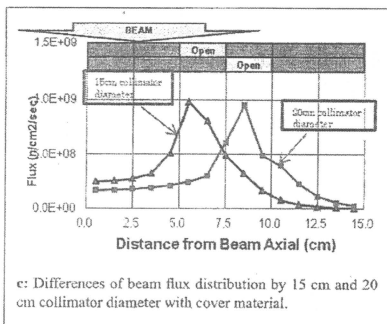
3. Results
We found that the 30 mm cover was sufficient (Figure b). However the 46 mm cover is naturally a better screen. The 15 mm thick cover is too thin, and the flux in the body of the mouse was double if compared with the cover 46 mm thick. The 30 mm cover is still flexible enough to be used as a shield for the mouse. Moreover, the importance of the shield thickness was confirmed for other cover materials, including the acrylic fiber and LiF.

A variety of collimator diameters and cover materials were examined to determine which would ensure the most uniform neutron beam irradiation of the animals. The best collimator diameter sets were determined to be 20 cm and 15 cm, and LiF cover material was 46 mm thick to protect the animals. After examining the different parameters, including irradiation conditions, duration of anesthesia, and driving time, the most suitable irradiation time was found to be 17 minutes, using the epithermal neutron beam.

4. Exclusive driving
Exclusive driving is indispensable to small animal irradiation, but it is difficult to perform under time limitations. In addition, it is necessary to start and stop the nuclear reactor, and this could cause potential problems with the staff security. Several issues remain to be solved regarding radioactivity management and the animal protection.
We should cooperate with the laboratory of university extension, a domestic study group about animal irradiation and the research reactor using section in JAEA which can be used domestically in Japan for animal irradiation studies. We had to overcome numerous problems in order to establish an effective animal irradiation device with the exclusive use of the research reactor.

b: Differences of beam flux by the thickness of cover material.

5. Conclusion

Unfortunately, the neutron outage has not been available for about the past 3 years. This paper focused on simulated neutron flux distribution and evaluated how various neutron doses can be delivered to different parts of the body in mice. When the JRR-4 facility, will be available again, we plan to verify the feasibility of the system and its applications for animal experiments.

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Development of a functional liposome modified a novel lipid analog for BNCT

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Abstract

We aimed at securing sufficient concentrations of ^{10}B in BNCT by developing a new drug delivery system. We have designed and developed a novel lipid analog and succeeded in using it to develop the new boron component liposome. It consisted of three different kinds of amino acid derivatives and two fatty acids, and could react directly with the peptide synthesized first on resin by Fmoc solid-phase synthesis. In this study, lipid analog conjugated with HIV-TAT peptide (domain of human immunodeficiency virus TAT protein) and boronophenylalanine (BPA) was synthesized and successfully incorporated into liposomes. This liposome showed in vitro the boron concentration of 156 times in comparison with BSH (sodium borocaptate) solution. This shows superior cell introduction ability of the liposome. Furthermore, as for this liposome including BSH solution experimented on by a similar method, improvement of the further boron concentration was developed.

1. Introduction

Boron neutron capture therapy (BNCT) is a tumor-selective radiation modality which depends on a sufficient cellular uptake of Boron (^{10}B) followed by irradiation with a beam of thermal or epithermal neutrons. ^4He and ^7Li particles are produced during the neutron capture reaction and damage DNA, which leads to cell killing. Regarding BNCT, the short radiation range of ^4He and ^7Li particles is decisive for the distribution of ^{10}B. Thus, successful treatment of cancer by BNCT requires the selective delivery of relatively large amounts of ^{10}B compound to malignant cells. The estimated boron concentration required for effective therapy is in the range of 20–30 μg ^{10}B per g tissue. However there have been no ideal boron compounds that fulfill the conditions of low toxicity, water solubility, and low distribution in normal tissue. Therefore, we aimed at securing sufficient concentrations of ^{10}B in BNCT by developing a new drug delivery system.

2. Materials and methods

2.1. Synthesis of lipopeptide

Lipopeptide conjugated with HIV-TAT peptide and boronophenylalanine (BPA) was synthesized on TGS-RAM resin by the Fmoc solid-phase synthesis method using an automatic peptide synthesizer (Shimadzu PSSM-8 Peptide Synthesizer Simultaneous Multiple) (**Figure 1**). Tryptophan residue was added at the N-terminus of HIV-TAT peptide as a fluorescence probe. BPA was coupled arbitrarily. Then, Fmoc-AEEA (9-fluorenylmethoxycarbonyl-8-amino-3,6-dioxaoctanoic acid, linker domain), 11 Fmoc-Asp-OtBu (hydrophilic domain), and
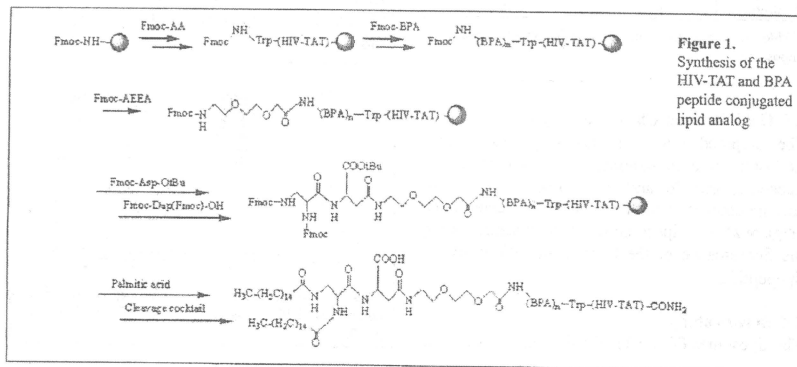
Figure 1. Synthesis of the HIV-TAT and BPA peptide conjugated lipid analog

Fmoc-Dap(Fmoc)-OH (glycero mimic domain) were coupled sequentially. Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), N-hydroxybenzotriazole (HOBt), and N-methyl morpholine (NMM) were used, respectively, for the peptide coupling reaction with 1.0, 1.0, and 1.5 equivalents based on amino acids. Fmoc amino acid and alkyl chain were used for resin in an equivalent of the excess of 7 and 6, respectively. Each coupling reaction was carried out for 30 min. The last condensation reaction with palmitic acid was carried out in a manual mode with the reaction progress checked by a ninhydrin test. De-protection and cleavage of resin were accomplished with a cleavage cocktail (10 mg/mL of 2-methylindole containing trifluoroaceticacid / H_2O/thioanisole/1,2-ethanedithiol/ethylmethyl sulfide/phenol = 82/5/5/3/2/3) for 16 hours at room temperature, then precipitated by adding a large amount of diethyl ether. After the drying procedure, we got a purpose thing.

2.2. Preparation of liposome

The lipid mixture prepared using the constant ratio (Table 1) was dissolved in organic solvent. It was prepared by the conventional lipid-film method. The resulting liposomes were extruded through polycarbonate membrane using an extruder, yielding the peptide- modified liposome.

	DSPC	Cholesterol	D S P E - PEG2000	B5-TAT-peptide
Plain liposome	50	50	-	-
PEG-liposome	47	47	6	-
B5-TAT5% PEG-liposome	45	45	5	5
B5-TAT10% PEG-liposome	42.5	42.5	5	10

Table 1. composition of B5-TAT-lpopeptide modified liposome.

2.3. Gel Filtration Chromatography

The prepared liposome was subjected to size exclusion chromatography, which separated the liposome, micelle and monomolecule fractions, and the content of the lipopeptide was determined (Figure 2). The liposome was then measured using the fluorescence of the tryptophan residue of the lipopeptide.

2.4. in vitro study

The liposomes (Table 1) added Colon 26 cell line

for two hours in Eagle's minimal essential medium, MEM, supplemented with 10% fetal bovine serum. And measured boron concentration by ICP-AES except a supernatant.
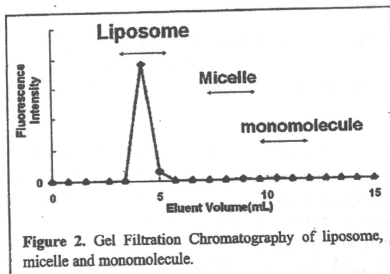

Figure 2. Gel Filtration Chromatography of liposome, micelle and monomolecule.

3. Results

3.1. Identification of lipopeptide

In the case of n=5 (B5-TAT), the C8-column used for high performance liquid chromatography (HPLC) analysis showed the main peak (retention time at 13.6 min) accompanied by the existence of some impurities (Figure 3a). However, the synthesis of conjugated lipopeptide attached to palmitoyl chain as an anchor domain proceeded very smoothly. HPLC analysis showed almost one peak, and ESI-TOFMS (electrospray ionization mass spectroscopy) showed m/z 3504 of the dehydration peak as an exact mass of m/z 3522. In addition, ^{1}H NMR analysis (JEOL JMN-AL400) also showed the structure of the lipopeptide; for example, the molar ratio of the TAT-peptide and palmitoyl moiety showed the correct proton ratio of the ortho position on the tyrosine residue (2H as a characteristic signal of the peptide at 6.62 ppm) to the methyl signal at 0.81 ppm in the alkyl chain end (6H as a characteristic signal of the lipid) using an integration value of ^{1}H NMR spectra (Figure 3b). The overall yield of the lipopeptide was greater than 70% based on the molar ratio of the amino group on TGS-RAM resin.
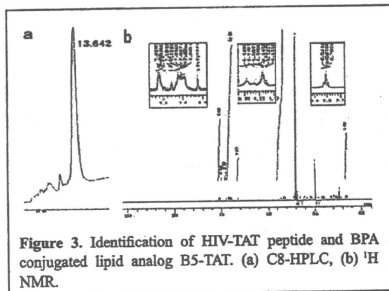

Figure 3. Identification of HIV-TAT peptide and BPA conjugated lipid analog B5-TAT. (a) C8-HPLC, (b) ^{1}H NMR.

3.2. Incorporated ratio of lipopeptide

The incorporation of lipopeptide into the liposome was examined. The methods are showed in Figure 2. Synthesized lipopeptide was incorporated into the liposome effectively. The incorporated ratios of lipopeptide to liposome are summarized in Table 2.

lipopeptide	Theoretical lipopeptide ratio (mol%)	incorporated ratio (%)
B5-TAT lipopeptide	5	66.1
	10	73.4

Table 2. The incorporated ratios of lipopeptide to liposome

3.3. Physical properties of liposomes

We examined the diameter of the liposomes with monodispersion and zeta-potential by means of dynamic light scattering measurement (Zetasizer Nano ZS, Malvern Instruments Ltd.) liposomes, which were composed of DSPC, cholesterol, DSPE-PEG and the lipopeptide, were prepared by the lipid-film method followed by extrusion. The diameters of the liposomes were shown to be about 100 nm. The positive charge of the peptide-modified liposome indicated the presence of basic peptide HIV-TAT on the liposome surface. The characteristics of liposomal formulations containing BPA conjugate peptide are summarized in Table 3.

DSPC:Chol:DSPE-PEG:B-TAT (molar ratio)	Particle size (nm, u%2)	Zeta-potential (mV)
Plain liposome	50 : 50 : 0 : 0	147	-40
PEG liposome	47 : 47 : 6 : 0	143	-15
B5-TAT lipopeptide liposome	45:45:5:5	120.3	17.5
	42.5:42.5:5:10	101.3	29.7
B5-FAT lipopeptide liposome	45:45:5:5	108.9	21
	42.5:42.5:5:10	147.8	26.4

Table 3. The diameters and zeta-potential of the liposomes

3.3. in vitro study

As a result, B5-TAT5% PEG liposome showed the boron concentration of 156 times in comparison with BSH solution. This shows superior cell introduction ability of the liposome.

Furthermore, as for the B5-TAT5% PEG liposome including BSH experimented on by a similar method, improvement of the further boron concentration was developed.

4. Discussion

We synthesized a new peptide lipid containing multiple BPA components and a TAT domain for use in a boron-containing liposome which can encapsulate a boron compound in its internal
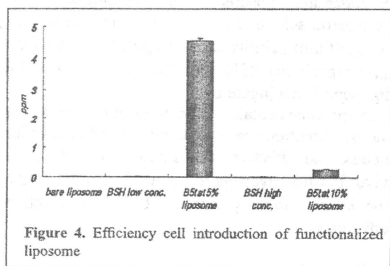

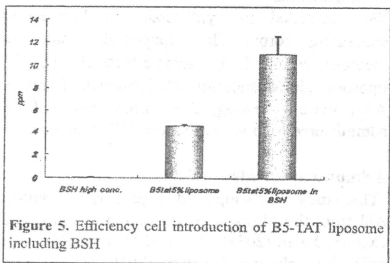Figure 4. Efficiency cell introduction of functionalized liposome

Figure 5. Efficiency cell introduction of B5-TAT liposome including BSH

water phase. The peptide lipid can be efficiently incorporated into liposomes that are 100 nm in diameter.

HIV-TAT was first developed from reverse transcriptase of HIV. It is a kind of protein transduction domain[3] which can introduce intracellular protein, deoxyribonucleic acid and macromolecule-containing liposome. Yagi et al. reported an in vitro anti-tumor effect of DOX encapsulated by TAT-modified liposome in 2007.[1] The TAT-conjugating liposome facilitated an in vitro gene expression as well as in vivo expression when the same liposome was locally injected[4].

Active targeting against tumor cells using TAT have been evaluated; however, there is no previous report involving a boron-containing TAT liposome or compound.

A sufficient concentration of boron is necessary for successful BNCT. Thus, a material with high boron content generally has an advantage.[2] Nakamura et al. developed a double-stranded boron cluster in 2004.[5] In the present study, the peptide lipid synthesized contains only 1 to 5 boron in a single molecule. However, our peptide lipid allows the number of boron to be increased up to n=12 or n=15.

In general, the hydrophilic charge of BSH in a boron-containing liposome has certain difficulty in encapsulating more BSH in the internal water phase of the liposome itself. There has been no previous report involving encapsulated BSH in the internal water phase within a boron liposome. Our peptide

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modification liposome of the hydrophilic charge is aspartic acid, and it shows high performance in terms of film stability and has a potential advantage in encapsulating BSH in the liposome in which the lipopeptide conjugate BPA.

The liposome containing the lipopeptide which give various functions be able to exhibit efficient cellar uptake and effective concentrations of ^{10}B for in vivo. Further investigation is needed to determine the *in vivo* toxicity and the boron introduction efficiency.

5. Conclusions

We succeeded in synthesizing a lipopeptide containing boron. This lipopeptide could be incorporated into the liposome effectively. And the liposome shows superior cell introduction ability. After toxicity testing, these liposomes will be administered to in vivo as a new BDS candidate.

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Biodistribution of BSH-encapsulated Boron-liposome in Mouse Glioma

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Abstract

Many boron reagents are synthesized and tested for BNCT, but no compounds except BSH and BPA have been
used for clinical BNCT. Boron containing liposomes are expected to be a new boron-delivery system in boron
neutron capture therapy (BNCT). We developed a new boron liposome, where sodium borocaptate (BSH) is
encapsulated into boron-conjugated lipid bilayer. The new boron liposomes show high blood retentivity (196.86
± 21.47 ppm at 24 h after administration) and low toxicity.

Keywords: liposome, BSH

1. Introduction

Boron neutron capture therapy is based on a
nuclear reaction of boron-10 and thermal neutrons.
Therefore, the successful treatment of cancer by
BNCT requires the selective delivery of large
amounts of ^{10}B compound to tumor cells. Previous
reports revealed that to achieve the anti-tumor effect
or tumor control effect by BNCT, the minimum
required boron concentration has to be 20 to 40
mg/g in tissue (ppm) with the thermal neutron flux
reactor source, which is equivalent to 10^9 atoms
of boron per each cell (Capala, 1996, Fairchild,
1985). We used the BSH and boronophenylalanine
(BPA) in clinical BNCT trials, but the doses of
these reagents exceeded 100 mg/kg, which is 10
to 100 times compared with other medical drugs
such as antibiotics or anti-cancer drugs. Ideal boron
compounds needs to show high tumor selectivity
and very low toxicity. Various approaches have
been taken to deliver boron compounds to the tumor
tissue, including the use of small boron molecules,
such as porphyrins, nucleosides and amino acids,
and boron-conjugated biological complexes,
such as monoclonal antibodies, epidermal growth
factors, carboran oligomers and dendrimers. The
recent promising approach is a liposomal boron
delivery system that meets the requirements for
BNCT (Mehta and Lu, 1996, Nakamura, 2008).
Two approaches have been investigated for
the incorporation of boron into liposomes: (1)
encapsulation of boron compounds into liposomes
and (2) incorporation of boron-conjugated lipids
into the liposomal bilayer. In this study, we report
the biodistribution of a new liposome, where BSH
is encapsulated into the boron-conjugated lipid
bilayer. The amount of boron that can be carried in
one liposome is increased by this method.

2. Materials and Methods

GL261 mouse glioma cells (5×10^6 / mouse) were
inoculated subcutaneously into the back of C57BL/6
mice. When the subcutaneous tumor had developed
into ca. 3-5 mm, tumor-bearing mice received new
boron-liposomes (1: 20 mg B/kg, 2: 40 mg B/kg)
i.v. via tail vein. To examine tissue boron uptake,
the animals (n=3 each) were sacrificed at 24, 36,
48 and 72 h after the administration of drug, and
samples of blood, brain, subcutaneous tumor, liver
and kidneys were obtained. A thermo-alumi-bath
(ALB-121, ASAHI TECHNOGLASS, Tokyo) was
used to solubilize the tissue samples. Then 0.8 ml of
69% nitric acid solution was added to tissue samples
(ca. 100 mg) which were subjected to thermolysis at
115°C for 2 h. Water was added to the lysed samples
to obtain a final volume of 5.0 ml and filtered
through the membrane filter to obtain the solution
for analysis. The quantity of boron was measured
by an inductively coupled plasma atomic emission
spectroscopy (ICP-AES, HORIBA, Japan).

3. Results and discussion

During 3 days of experimentation, adverse
reactions were not observed with the given drug
concentration. Figure 1 shows the time course of
boron concentration in various tissues of tumor-
bearing mice after injecting the new liposome drug
(a: 20 mg B/kg, b: 40 mg B/kg). Since tumors in
20 mg administration group were too small, the
boron concentration in the tumor tissue could not
be measured. Tumor/blood ratio was 1.13 at 24 h
and 0.28 at 48 h. Tumor/brain ratio was 979.28 at
48 h. The blood boron concentration at 24 h after
the drug administration was higher in comparison
with BSH (statistical analysis was not performed).
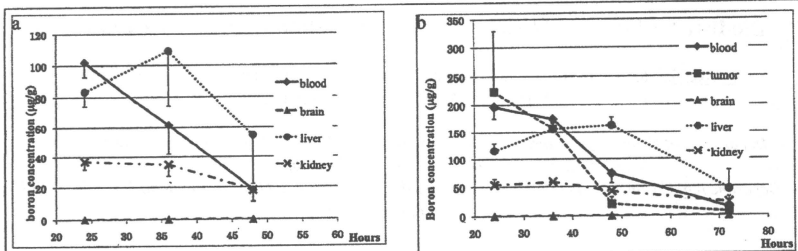Using the model of nude mice carrying oral

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Fig. 1 Time course of boron concentration in various tissues   a: 20 mg B/kg, b: 40 mg B/kg

squamous cell carcinoma, Obayashi et al. reported that blood boron concentration was 3.29 ± 1.38 ppm at 3 h after the administration of BSH at a dose of 75 mg/kg (Obayashi, 2004). Blood boron concentration was maintained over 50 ppm at 48 h after the administration, revealing the stability of new liposome in blood. In contrast, tumor boron concentration was less than 20 ppm, which is low and insufficient for BNCT.

Our new boron liposomes have two boron components: one is BSH encapsulated within the inner water phase of nano-capsules, and the other is represented by boron-containing lipids, which form the lipid layers. Liposome infiltrated into the tumor stroma and capsules were endocytosed by tumor cells. Then lipid layer was degenerated and inner water phase collapsed into the cytoplasm. Boron-containing lipid could be retained into the tumor cells, but water soluble BSH would be promptly eliminated by the diffusion. ICP boron measurement cannot distinguish between the boron from liposome circulating in the blood and the boron escaped from the tumor cells.

Liposome containing polyethylene glycols (PEGs) makes the environment around liposomes hydrophilic and plays the role of a stabilizer in aqueous phase. But excessive amounts of PEGs prevented endocytosis by tumor cells. The appropriate compounding rate of PEGs needs to be defined.

In our study, blood retentivity was good, but tumor/blood ratio was not high. In future studies, we also need to confirm the neovascularization in the subcutaneous tumor model used in this study. As solid tumors are characterized by high vascular permeability, particulate liposomes can easily extravasate into the tumor stroma.

Thus, long-circulating liposomes can exploit the 'enhanced permeability and retention' effect for the preferential extravasation from tumor vessels.

4. Conclusions

We studied the biodistribution of a new boron-liposome, where BSH is encapsulated into the boron-conjugated lipid bilayer. The drug showed low toxicity and high blood retentivity.

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特 集　ドラッグデリバリー

中性子捕捉療法におけるドラッグデリバリーシステムの応用

Application of Drug Delivery System to Neutron Capture Therapy for Cancer

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Tumour cell destruction in boron neutron-capture therapy (BNCT) is due to the nuclear reaction between ^{10}B and thermal neutrons ($^{10}B + {}^1n \rightarrow {}^7Li + {}^4He$ (α) + 2.31 MeV (93.7 %) / 2.79 MeV (6.3 %)). The resulting lithium ions and αparticles are high linear energy transfer (LET) particles which give high biological effect. Their short range in tissue (5 - 9μm) restricts radiation damage to those cells in which boron atoms are located at the time of neutron irradiation. BNCT has been applied clinically for the treatment of malignant brain tumors, malignant melanoma, head & neck cancer and hepatoma etc, recently. Sodium borocaptate(Na$_2{}^{10}B_{12}H_{11}$SH ; BSH) and boronophenylalanine (^{10}BPA) are currently being used in clinical treatments. These low molecule compounds are easily clearanced from the cancer cells and blood , so high accumulation and selective delivery of boron compounds into tumor tissues & cancer cells are most important to achieve effective BNCT and to avoid damage of adjacent healthy cells. To achieve the selective delivery of boron atoms to cancer cells, drug delivery system(DDS) becomes an attractive intelligent technology as targeting and controlled release of drugs.

Keywords: Neutron Capture Therapy(NCT), boron compound, gadrinium compound, Drug Delivery System (DDS).

1 緒言

　中性子捕捉療法 (Neutron Capture Therapy;NCT) は熱中性子線をボロン (^{10}B) 化合物あるいはガドリニウム(Gd)化合物に照射することにより得られる重電荷粒子 (α 線, Li) あるいは電子線を使用する物理化学的な癌治療法である。α 線, Li 核のエネルギー飛程は 10μm であり，癌細胞にボロン原子を選択的に集積させることができれば正常細胞に障害を与えず理論的には細胞単位での癌特異的治療が可能である。つまり BNCT は副作用が無く，癌患者の QOL を改善することが可能であるが，そのためにはボロン化合物の癌細胞選択的そして高効率デリバリーシステムが必要不可欠である。ボロン化合物を患者に投与時に最小毒性を考慮して治療することが，患者への負担軽減及び経済負担の軽減となりうると思われる。副作用を軽減させるための投与量の低減および負担金額の軽減という問題を克服するために，腫瘍集積性向上を計るべく ^{10}B 化合物のリポソーム製剤化や高分子製剤化が国内外で活発に展開されている。現在, BNCT は悪性脳腫瘍, 悪性黒色腫, 頭頸部癌に臨床試験が開始されており，我々は，難治

性癌である多発性肝細胞癌，進行・再発乳癌などへの適応拡大を目指している。

　癌細胞により発現している受容体の数は多いものから少ないものまで多様であり，十分な量のボロン化合物を取り込ませるには，それぞれの受容体において反応するボロン化合物を増加させる必要がある。そのためにも，多くのボロン原子を結合あるいは封入した受容体選択的なリガンドキャリアーを含めたドラッグデリバリーシステム(DDS)を中性子捕捉療法へ応用する必要性が生じてきている。モノクローナル抗体，リポソーム，ポリマーミセル，高分子ポリマーなどが過去３０年にわたり選択的薬剤キャリアーとして研究されてきている。これらのキャリアーは低分子のものから高分子のものまでのサイズの幅を持っている。モノクローナル抗体(mAb) 療法が脚光を浴びてきた背景には，非免疫原性な mAbs（すなわちヒト抗 mAb 抗体を生み出さない mAbs）の開発，標的とされる抗原の安定性と投与経路の選択などを含むいくつかの要因に依存している。分子標的薬：セツキシマブは EGFR と結合し，リガンドにより誘発されたチロシンキナーゼ起動を妨害していて，レセプター内面化を刺激しているリガンドを防いでいる。さらにセツキシマブは，アポトーシスを誘発して，血管形成も妨いでいる。また効果増強のために，mAbs は抗癌剤，毒素または放射

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性同位元素の複合体としての活用にも展開できる。た
とえば、脳組織に対しては血液脳関門が抗体の侵入を
防ぐので、mAb療法は、むしろ腫瘍内局注で用いられ
ることも多い。その場合は、カテーテルの使用やCED
法による投与が用いられ、全身的な副作用の軽減にも
つながっている。また、腫瘍内投与は腫瘍摘出後にも
応用できる。

　ナノキャリアーにおいては、静脈投与により全身の
大循環系にはいり血流に乗ることにより血中滞留時間
を延長させる狙いがある。この目的のためにポリエチ
レングリコール(PEG)は被覆剤として良く用いられる。
この結果PEGで被覆したキャリアーは細網内皮系の
マクロファージによる貪食作用を回避でき、血中滞留
性を増加させることができている。この滞留時間の増
加は、癌や炎症部位における血管透過性の増加および
漏出を可能にするために必要である (enhanced
permeability and retention effect (EPR効果)。リポソーム
は、簡単な調整法、良い生体適合性と生物分解性、低
い毒性と商業的な有効性のため、ワクチン、治療薬と
ホルモン類の送達のためのキャリヤーシステムとして
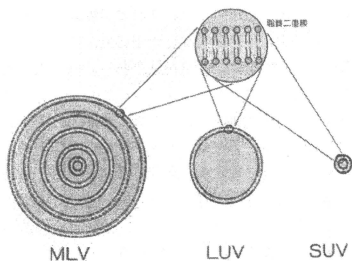多く使われている (Fig.1, 2)。

Fig.1　リポソームを用いたターゲティング
リポソームは脂質二重膜により構成される小胞体で、
水溶性・脂溶性両薬物のキャリアーとして用いられる。
調整法や調整に用いる脂質を選択することにより粒子
径・表面電荷・硬さ等をコントロールできる。リポソ
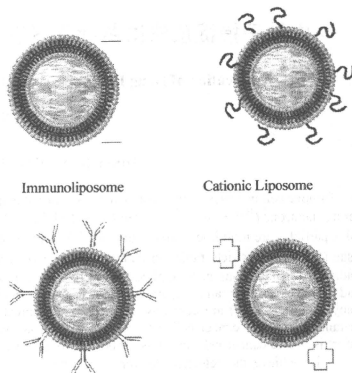ームは形態上、多重層リポソーム(MLV)、大きな一枚
膜リポソーム(LUV)、小さな一枚膜リポソーム(SUV)
の3種類に大別される。

Fig.2　代表的なリポソーム
Conventional liposome：最初にBanghamが合成した基
本的なリポソーム, Long circulating liposome：血中滞留
性を高めるためにポリエチレングリコール(PEG)など
を表面に結合させてあるリポソーム（リポソーム表面
のPEGなどの水和層がマクロファージによるオプソ
ニン効果を抑制する), Immunoliposome：Targetingを行
うためにリポソーム表面に抗体を結合させ標的への結
合能を高めたリポソーム, Cationic liposome：遺伝子デ
リバリーを行うリポソーム　(癌細胞の表面はanionic
になっており、cationicなリポソームは結合しやすい)

　さらにボロンナノキャリアーの治療効果を増大さ
せるために、標的部位においては被覆剤を取り除きま
たはリガンドを用いた癌細胞への結合が必要になる。
また、封入している溶液および薬剤の溶解度の組成だ
けでなく、生体における薬剤の放出率、薬剤の吸収率
を含めた標的化、薬剤デリバリーを制御しなければな
らない。

　この章では、現在までにNCTにおいて開発されて
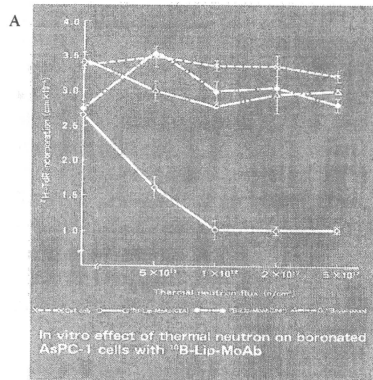きたデリバリーシステムに関して紹介する [1]。

2　ドラッグデリバリーシステム各論

2.1　モノクローナル抗体

　Barthらは、結腸直腸癌に対する中性子捕捉療法にお

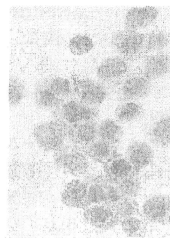いて,ボロン化合物の結合したモノクローナル抗体17-1Aを開発し,ホウ素送達系のためのモノクローナル抗体の応用を最初に報告した [2]。

高橋・柳衛らは,ホウ素原子(^{10}B)を AH-66 肝癌細胞に送達するための BDS として,肝細胞癌が分泌するα-フェトプロテイン(AFP)に対して,モノクローナル抗体 (mAb) の応用を報告した [3,4]。MAb は,N-succinimidyl 3(2-pyridyldithio) propionate (SPDP)を用いて ^{10}B-合成物($Cs_2{}^{10}B_{12}H_{11}SH$)を結合しホウ素化された。抗体分子に接合される ^{10}B 原子数は,約 1240 であった。ホウ素化 α-AFP MoAb を反応させた AH-66 細胞において $11X\ 10^9\ {}^{10}$B 原子が AH-66 細胞表面および細胞内に存在すると考えられた。熱中性子による照射後,boronated された AH-66 細胞は,取り込んだ ^{10}B の数に比例して,in vitro で細胞障害効果を認めた [3]。さらに,α-AFP mAb(2mg/ml)に 50mM の ^{10}B 化合物を接合することによって,ホウ素化 α-AFP MoAb を調整後,3.0mg の ^{10}B 結合 α-AFP mAb の静脈投与 12, 24, 72 と 120h 後に得られる腫瘍組織のホウ素濃度は,11.10 +/- 3.12 (SD, n = 6). 29.30 +/- 5.11, 33.02 +/- 11.8, and 12.91 +/- 5.62 ppm であり,有効なボロン原子を腫瘍細胞に送達できる可能性を示した [4]。Michael らは,マレイミド基を用いてボロン化合物を mAb B72.3 に結合させ,生理的に安定したボロン-抗体複合体を作製した。LS174-T 腫瘍担癌マウスに静脈投与後,^{125}I でラベルされた複合体は腫瘍選択的に集積し,腫瘍細胞1個に対して 10^6 個のボロン原子を送達できることを示した [5]。Barth らは,脳腫瘍の BNCT のためのホウ素送出キャリアーとして,分子標的治療薬 Cetuximab (IMC-C225) を評価した。Cetuximab は,上皮細胞増殖因子受容体(EGFR)の野生型と変異体 vIII isoform に向けられる mAb である。ホウ素化された Cetuximab (C225-G 5-B(1100))の腫瘍内投与 24 時間後に,F98(EGFR)および F98(WT)神経膠腫の平均ホウ素濃度は,92.3+/-23.3μg/g と 36.5+/-18.8μg/g であった。Convection-enhanced delivery(CED)により投与された C225-G 5-B(1100)を用いた BNCT においては,平均生存期間 (MST) が 45+/-3d であり,非治療群 25+/-3d と比較して優位に延長していた。さらに BPA の静脈投与を併用した BNCT 群においては 59d とさらに延長した。このように抗体を用いたボロン送達システムと新規のボロン結合低分子化合物の併用により神経膠腫への BNCT の新しい展開が期待できる [6]。

2.2 リポソーム

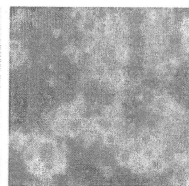柳衛らは,^{10}B 化合物を封入させたイムノリポソームが,BNCT において腫瘍細胞を標的とする ^{10}B 原子の選択的で効率的なキャリヤーの働きをすることができることを最初に報告した。大腸癌や膵臓癌など腺癌で分泌される胎児性抗原のひとつである Carcinoembrionic antigen(CEA)に対するモノクローナル抗体(2C-8)を作製した。柳衛は,ボロン化合物を封入した封入抗 CEA イムノリポソームを CEA 産生性ヒト膵癌細胞と反応させ,イムノリポソームは高濃度の ^{10}B 原子を腫瘍細胞に送達でき,熱中性子による照射の後,($1x\ 10^{11}$ - 1×10^{13}n/cm^2),ホウ素化された AsPC-1 細胞は,対照群と比較して ^{3}HTdR の取り込み減少を呈し細胞障害性効果を示した (Fig.3) [7]。

In vitro effect of thermal neutron on boronated AsPC-1 cells with ^{10}B-Lip-MoAb
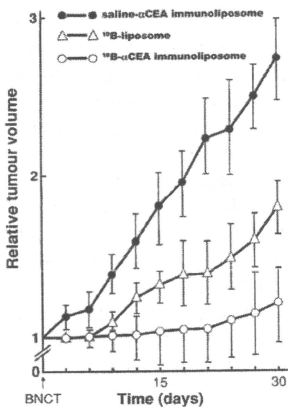
D

Fig.3 ボロン封入イムノリポソームを用いた中性子捕捉療法による腫瘍増殖抑制効果：ボロン封入抗CEA モノクローナル抗体結合イムノリポソームは癌胎児性タンパクである CEA を発現しているヒト膵臓癌細胞株 AsPC-1 と選択的に反応する。ボロン封入抗CEA MoAb結合イムノリポソームと反応した癌細胞はＢＮＣＴにより選択的に細胞障害効果を誘導できた (A : BNCT の細胞障害効果, B:免疫染色, C : 免疫蛍光染色) (Yanagie et al: BJC, 1991)。さらに AsPC-1 移植担癌マウスにおいてボロン封入抗 CEA MoAb 結合イムノリポソームを腫瘍内投与しＢＮＣＴにより選択的に腫瘍増殖抑制効果を誘導できた (Yanagie et al: BJC, 1997) (D)。

さらに，ボロン化合物封入抗 CEA イムノリポソームの腫瘍内投与群において BNCT を用いて選択的な腫瘍増殖抑制効果を認めた (Fig.3) [8]。また柳衛らは，10B を含有する多重膜リポソームをヒト乳癌細胞と反応させ熱中性子捕獲反応によって，乳癌細胞の増殖を抑制した [9]。

Shelly らは，平均直径 70nm 以下の SUV リポソームを開発した。このリポソームは合成リン脂質(distearoylphosphatidylcholine)とコレステロールから構成され水溶性イオンホウ素合成物と $B_{20}H_{18}^{2-}$ のphotoisomer を内封している。腫瘍内ホウ素濃度は 15μg/g，および腫瘍/血液比が 3 以上を維持できたと報告している[10]。Feakes らは，$[B_{20}H_{17}SH]^{4-}$ の Na 塩を封入した SUV リポソームを開発した。EMT6 乳癌の皮下移植モデルにおいて静脈投与 48 時間後，腫瘍内ホウ素濃度は 46.7μg/g，および腫瘍/血液比が 7.7 に到達できたと報告している[11]。Hawthone らは，多面ボランNa3 [a2-$B_{20}H_1$-$NH_2CH_2CH_2NH_2$ を含んでいる SUVリポソームを報告した。彼らも担癌マウスモデルにおいて静脈投与 30 時間後，腫瘍内ホウ素濃度は 45μg/g，および腫瘍/血液比が 9.3 に到達できたと報告している[12]。

Mehta らは，BSH が封入された DPPC/CHOL とポリエチレングリコール(PEG)のモル比 1:1 で平均直径100-110nm の PEG リポソームを調整した。PEG リポソームを 1.88 mg リン脂質/マウスおよび 3.5-5.8mg のBSH/kg マウス体重にて静脈投与し，BSH 水溶液投与群と比較して，BSH の血中滞留性の改善を認めた。また 24 時間後の BSH の血中残存量が 19%であり Bareリポソーム 7%と比較して優位に滞留していた。このため，PEG リポソームは BDS としてホウ素の腫瘍への送達増強効果が期待できると思われる [13]。

柳衛・丸山らは，BSH を封入した PEG-リポソーム(DPPC/コレステロール/DSPC-PEG2000)からなる送達系を開発しました。ヒト膵臓癌細胞 AsPC-1 移植担癌マウスに対して 10B-PEG liposome の静脈注射後，熱中性子照射にて腫瘍増殖抑制を認めた [14]。

石田・丸山らは，トランスフェリン（TF）結合PEGliposome が標的細胞に抗癌剤やプラスミド DNAの細胞質内への targeting に役立つことを示唆した。TF-PEGliposome は，リポソームにつき約25 の TF 分子を結合しており，癌細胞表面のレセプターと結合するエンドサイトーシスによって細胞に内在化されることが明らかになった[15]。丸山と柳衛のグループは，BSHを封入した unilamellar TF-PEGliposome を報告した。TF-PEGliposome を 35μg/kg の 10B 濃度で投与した場合に，細網内皮系（RES）による取り込み抑制と血中対流性の向上を認めた。TF-PEG リポソームは，腫瘍において高い 10B 濃度を維持した。腫瘍組織における 10B濃度の高い保持は，遊出する TF-PEG リポソームの細胞内取り込みが TF レセプターとレセプターによって介在されるエンドサイトーシスによって起こることを示している。静脈投与 72 時間後の腫瘍/血液比は 6.0を示し，熱中性子照射により腫瘍増殖抑制効果を認めた (Fig.4)[16, 17]。
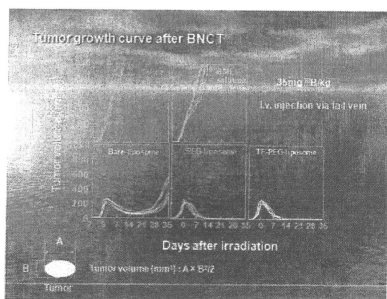
Fig.4 ボロン封入リポソームの中性子捕捉療法治療効果 ボロン化合物をそれぞれのキャリアーで Colon 26 担癌マウスに静脈投与後，熱中性子を照射し，治療効果を検討すると，PEG 化したリポソーム用いた群において著明な腫瘍増殖抑制効果を認めた。

小倉・小林・柳衛らは，担癌動物における ^{10}B 原子の正確な部位同定と定量のため α トラックディテクターである CR39 を用いた中性子ラジオグラフィー（NCAR）テクニックを報告した（Fig.5）[18]。癌に対する BNCT において効果的 ^{10}B キャリアーを選ぶために NCAR テクニックを適用することができる。また，冷中性子を用いてイメージの品質の改善ができることも見出した[19]。

Pan らは，ＢＤＳとして葉酸結合-PEG リポソームを報告している。彼らは，Na2B12H11SH と Na3(B20H17NH3)の二つのホウ素化合物を封入したリポソームを開発している。葉酸レセプターを発現しているヒト扁平上皮癌 KB 細胞に対して反応させると 10B 濃度として 1584 μg/ 10^9 cells まで上昇させることができ，この値は非 targeting リポソームの 10 倍の濃度であった。さらに担癌マウスモデルにおいても静脈投与 24 時間後に 85 μg/g の 10B 濃度に達することができた。これらの機序は葉酸-葉酸レセプターを介するエンドサイトーシスによるものである[20,21]。Stephenson らは，Na3 (B20H17NH3) 封入葉酸標的リポソームを開発した。葉酸レセプターは，90%以上の卵巣癌を含むさまざまな腫瘍で増幅されている。葉酸レセプター陽性 KB 細胞皮下移植モデルにおいて 24 時間後に腫瘍内ホウ素濃度のピークに到達し投与後 120 時間までの腫瘍/血液比の持続的な増加を報告した[22]。Pan らは，

(a) ^{10}B Bare-liposome
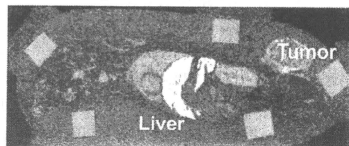
(b) ^{10}B PEG-liposome
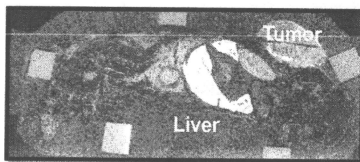
(c) ^{10}B TF-PEG-Liposome
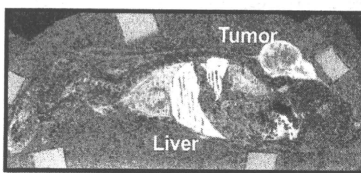
Fig.5 ボロン封人 PEG リポソーム静脈投与後の担癌マウスにおける中性子ラジオグラフィー Colon 26 腫瘍においてはボロンの集積が，単リポソーム(A)よりも PEG 化したリポソーム(B)において増加している。またトランスフェリン結合 PEG リポソーム(C)においてさらに増加している。

EGFR 陽性神経膠腫細胞にホウ素合成物を送達しＢＮＣＴを行うために分子標的治療薬であるモノクローナル抗体；セツキシマブを応用しセツキシマブ-免疫リポソームを作製した。セツキシマブをリポソームに取り込むために，免疫リポソームは新しいコレステロールベースの膜アンカー（maleimido-PEG-コレステロール（Mal-PEG-Chol））を使用して合成された。非特異的なヒト IgG-免疫リポソームと比較して，cetuximab-免疫リポソームを用いた場合，EGFR 陽性 F98EGFR 細胞ではホウ素の細胞内取り込みが約 8 倍増加した[23]。Wei らも，消化器癌や乳癌に発現している HER2

に対して，陽性癌細胞にホウ素合成物を送達しＢＮＣＴを行うために分子標的治療薬であるモノクローナル抗体；Trastuzumab を応用し，水溶性ホウ素化アクリジン（WSA）封入 Trastuzumab -免疫リポソームを作製した。Trastuzumab は PEG-DSPE-NHS の末端部に反応させリポソームに結合している。このリポソームは SK-BR-3 細胞の HER-2 レセプターに選択的に結合し，24 時間後の細胞内ホウ素濃度が 132ppm に到達した。ホウ素化合物である WSA は細胞質内に留まり長期のホウ素濃度を維持できることもわかり，Trastuzumab-リポソーム-WSA は，BNCT のための有力な薬物送達システムと考えられた[24]。

Thirumamagal らは，癌細胞の受容体を標的とするホウ素化リポソーム作製において脂質二分子層構成要素として，3 つの新しい carboranyl コレステロール誘導体を合成した。これらのホウ素化コレステロール誘導体を用いて葉酸レセプター(FR)や血管内皮成長因子 receptor-2 (VEGFR-2) を標的とするリポソームを作製した。FR を過剰発現させている KB 細胞と VEGFR-2 を過剰発現させている 293/KDR 細胞はホウ素化された FR と VEGFR-2 を標的とするリポソームと反応後，それぞれのレセプター選択的に反応を認めた[25]。

Peacock らは，ホウ素を含む脂質として新規のコレステロールカルボラン接合体 (BCH) を報告しました。この BCH を用いた PEG リポソーム製剤の BCH の細胞取り込みが ^{10}B：45.9μg/g 細胞であり，ＢＮＣＴに有効な濃度であった[26]。中村らは，新規の nido-カルボラン脂質を開発し，安定したリポソームを合成できることを報告した。宮島・中村らは nido-カルボラン脂質を用いてトランスフェリン結合 PEG-リポソーム(Tf (+) -PEG-CL-Lip)の合成に成功した。ホウ素化 nido-カルボラン脂質は，heptadecanol から合成された。この Tf (+) -PEG-CL-Lip を担癌マウスに静脈投与後 (7.2μg/kg body)，腫瘍内ホウ素濃度は 22ppm であり，BNCT により長期生存を認めた[27,28]。

Ristori らは，カルボランが BNCT の効率的なホウ素送達体であり，さらに，正荷電脂質として DOTAP およびヘルパー脂質として双性イオン脂質 DOPE を用いてカチオニックリポソームを合成しＢＮＣＴへのホウ素送達の可能性について報告している[29]。柳衛らは，BSH を混和させたカチオニックリポソーム溶液 (COATSOME-EL-C01) が腫瘍内注射の 3〜6 時間後に AsPC-1 腫瘍でホウ素合成物の保持を示すと報告した [30]。

中井らは，HVJ-リポソーム（HVJ-E と融和するリポソーム）に高濃度のホウ素を封入できると報告した。HVJ（HVJ-E）ベクターシステムは，不活性センダイウイルスに基づく新しい融合によりもたらされる遺伝子送達系である（HVJ;センダイウイルス）。BSH 封入 HVJ-E リポソームと 60 分のインキュベーションの後の細胞内 10B 濃度が BHK-21 細胞では 24.9μg/g，SCC VII 細胞では 19.4μg/g であることを示した。HVJ-E は腫瘍細胞膜と融合して，速くホウ素化合物を送達することができるので，BNCT [65] に，リポソームを含む HVJ-E によって媒介されるホウ素送達系を適用することも進められている[31]。

2.3 ポリマー

Shukla らは，細網内皮系による取り込みを減らすことにより BNCT のために必要な ^{10}B の腫瘍への集積を増加させるため，腫瘍の葉酸レセプターを標的として葉酸を端末に結合した 13 個の decaborate，PEG(2000) と PEG(800) を結合させた polyamidoamine dendrimers を合成した。このポリマーは葉酸レセプター依存的に取り込みを認めた [32]。柳衛らは，ボロン化合物を結合したＰＥＧアルブミン複合体を作成し，ヒト膵臓癌細胞 AsPC-1 との反応性を検討した。このボロン結合ＰＥＧアルブミン複合体は γ-maleimidobutyryl-oxysuccinimide (GMBS) を用いてボロンを結合させている。FITC を指標にしたボロン結合ＰＥＧアルブミン複合体はを細胞に反応させると AsPC-1 細胞の蛍光強度は培養時間とともに増加することがわかった。単核球による貪食作用はＰＥＧアルブミン複合体を用いることにより抑制された。最近，癌細胞におけるアルブミン受容体の発見にともない，ボロン結合ＰＥＧアルブミン複合体において結合させるボロン化合物の量を増やしたり，さらに反復投与することにより有効濃度まで上昇させることが期待できる[33]。

肝細胞癌は，手術，化学療法，放射線療法を駆使しても治癒が困難な癌のひとつである。ヨード化ケシ油（商品名：リピオドール）は，脂質自体が選択的に肝臓癌に沈着する性質を有しており，肝臓癌の検出および治療に有効であることが報告されている。鈴木らは，ラットの肝腫瘍モデルにおいて BSH とリピオドールのエマルションを肝動脈より投与し有効性を証明した。肝腫瘍におけるボロン濃度および腫瘍 / 正常肝組織ボロン濃度比(T/L)は，BSH リピオドールエマルション投与後 1，6，12 時間後においてそれぞれ 479.2 ppm：

4.0, 197.3 ppm: 14.9, 96.5 ppm: 6.6 であった[34]。さらに、鈴木らは動注塞栓用デンプン微粒体（DSM）を用いた BSH の動脈内投与法を報告している[35]。

柳衛・東らは、肝臓癌に対する肝動注法を用いたホウ素封入WOWエマルションの応用について報告した。WOWエマルションでは、シラス多孔質ガラス膜を用いた二段膜乳化法により、従来のリピオドールを均一なサイズと封入効率を格段に高めることができる。VX-2 ウサギ肝腫瘍に対する肝動注モデルにおいて、WOW エマルションによる VX-2 腫瘍内の ^{10}B 化合物の集積および保持能力は、従来型の ^{10}BSH-Lipiodol エマルションまたは 10BSH 溶液と比較して優れていた。WOW エマルションの電子顕微鏡を用いた解析により、構成脂質の microdroplets の細胞内集積も認められ、腫瘍への動注ホウ素送達キャリアーとしての可能性を見出した（Fig.6, 7）[36]。
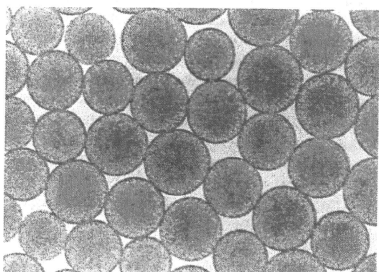

Fig.7　中性子ラジオグラフィーによるボロン(BSH)封入 WOW エマルションによる腫瘍集積性の確認
VX-2 肝腫瘍モデルにおいて BSH 封入 WOW エマルションを肝動注し、投与 3 日、7 日後の腫瘍集積性を検討すると、BSH 溶液と臨床で用いられているリピオドールとの混合液投与群と比較して、BSH 封入 WOW エマルション投与群は、優位に腫瘍内の貯留性を示した。

Fig.6　シラス多孔質ガラス膜を用いて二段膜乳化法により作成したWOWエマルションWOWエマルションにおいてはサイズが均一でありボロン水溶液を内封できる。我々は、^{10}BSH 封入 WOW エマルションを作成し、肝臓癌に対して選択的肝動注を施行し中性子捕捉療法を計画中である。

Wu らは、分子標的治療薬である抗上皮細胞増殖因子(EGF)抗体：セツキシマブ（IMC-C225）をホウ素化 polyamidoamine dendrimer に結合させた。脳腫瘍一つである神経膠腫は EGFR を高発現しており、マウスモデルでは CED 法により腫瘍内に投与されたホウ素濃度と腫瘍/血液比の増加とそれに伴うＢＮＣＴによる生存日数の延長を報告している [37]。

鍋田・福森らは、ガドリニウム中性子捕捉療法に向けて、粒径 63nm のナノ粒子（Gd-nanoGR）を合成した。この Gd-nanoGR の静脈投与 6 時間後の腫瘍内ホウ素濃度は 109μg/g に到達できたと報告している [38]。福森らは、0.5%の 10kDa のキトサンを含んだ Gd 封入ナノエマルションを開発した。このナノ粒子は構成成分中22%のGdを含んでおり、サイズは155nmであり、BNCT により B16F10 悪性黒色腫細胞に対して強い腫瘍増殖抑制を示した [39]。

3 結言

中性子捕捉療法は、生体への影響が少ない熱中性子・熱外中性子とボロン・ガドリニウム化合物との反応を利用した新規の放射線化学治療ということができる。より生体内の正常組織への副作用を軽減させ、かつ治療効果を上げるために本編でのべたような DDS の応用が望まれる。今後、テクノロジーの進歩に伴ってより高精度の腫瘍集積性のデリバリーキャリアーが開発されると思われるが、臨床基準の GMP グレードの製剤まで開発を進め早期に臨床試験への展開が期待できるトランスレーショナルリサーチのシステム作りも重要な課題と考えられる。

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Cancer Research

Circadian Rhythm of Transferrin Receptor 1 Gene Expression Controlled by c-Myc in Colon Cancer–Bearing Mice

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Abstract

The abundance of cell surface levels of transferrin receptor 1 (TfR1), which regulates the uptake of iron-bound transferring, correlates with the rate of cell proliferation. Because TfR1 expression is higher in cancer cells than in normal cells, it offers a target for cancer therapy. In this study, we found that the expression of TfR1 in mouse colon cancer cells was affected by the circadian organization of the molecular clock. The core circadian oscillator is composed of an autoregulatory transcription-translation feedback loop, in which CLOCK and BMAL1 are positive regulators and the *Period* (*Per*), *Cryptochrome* (*Cry*), and *Dec* genes act as negative regulators. TfR1 in colon cancer–bearing mice exhibited a 24-hour rhythm in mRNA and protein levels. Luciferase reporter analysis and chromatin immunoprecipitation experiments suggested that the clock-controlled gene *c-MYC* rhythmically activated the transcription of the *TfR1* gene. Platinum incorporation into tumor DNA and the antitumor efficacy of transferrin-conjugated liposome-delivered oxaliplatin could be enhanced by drug administration at times when TfR1 expression increased. Our findings suggest that the 24-hour rhythm of TfR1 expression may form an important aspect of strategies for TfR1-targeted cancer therapy. *Cancer Res; 70(15); OF1–9. ©2010 AACR.*

Introduction

In mammals, the master pacemaker controlling the circadian rhythm is located in the suprachiasmatic nuclei of the hypothalamus (1). Regulation of circadian physiology relies on the interplay of interconnected transcription-translation feedback loops. The BMAL1/CLOCK complex activates clock-controlled genes, including *Per*, *Cry*, and *Dec*, the products of which act as repressors by interacting with BMAL1/CLOCK (2–5). This mechanism also regulates the 24-hour rhythm in output physiology through the periodic activation/repression of clock-controlled output genes in healthy peripheral tissue and tumor tissue (6, 7).

Transferrin receptor 1 (TfR1) is involved in the uptake of iron into cells through the binding and internalization of transferrin, and its regulation by intracellular iron levels has assisted in the elucidation of many important aspects of cellular iron homeostasis (8, 9). Iron is important for

metabolism, respiration, and DNA synthesis. Thus, TfR1 is expressed not only in normal healthy cells but also in malignant tumor cells (8, 10). Recently, another TfR-like molecule named TfR2 has been recognized and investigated (11, 12), but the exact function of TfR2 remains unclear (8). It has been reported that the expression of TfR1 in mammary epithelial cells exhibits a significant 24-hour rhythm (13). Such rhythmic variation in TfR1 expression seems to affect its iron uptake function resulting in time-dependent changes in the internalization of iron-loaded Tf. However, it is not clear if the expression of TfR1 in colon cancer cells shows a significant 24-hour rhythm.

Many of the pharmacologic properties of conventional drugs can be improved through the use of an optimized drug delivery system (DDS), which includes particular carriers composed primarily of lipids and/or polymers (14). The high expression of TfR1 in tumor can potentially be used to deliver cytotoxic agents into malignant cells, including chemotherapeutic drugs, cytotoxic proteins (8), and Tf-coupled polyethylene glycol (Tf-PEG) liposomes were designed as intracellular targeting carriers for drugs by systemic administration. In fact, Tf-PEG liposomes encapsulating a platinum (Pt)-based anticancer drug, oxaliplatin, can increase its accumulation in tumor masses (15, 16). On the other hand, daily rhythmic variations in biological functions affect the efficacy and/or toxicity of drugs: a large number of drugs cannot be expected to have the same potency at different administration times (7, 17). However, it is unclear what influence the rhythmic expression of TfR1 has on the pharmacokinetics/pharmacodynamics of transferrin targeting liposomes.

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In this study, we found that the circadian expression of c-Myc, which is controlled by the circadian clock, affects TfR1 gene transcription in colon cancer cells. The levels of TfR1 mRNA and protein exhibited a 24-hour oscillation in tumor cells implanted in mice. Thus, to evaluate the rhythmic function of TfR1 and the utility for TfR1-targeting cancer therapy, we investigated how the rhythmic variation in TfR1 production influenced the pharmacologic efficacy of TfR1-targeting liposomal DDS.

Materials and Methods

Animals and cells

Seven-week-old male BALB/c mice (Charles River Japan) were housed with lights on from 7:00 a.m. to 7:00 p.m. at a room temperature of $24 \pm 1°C$ and a humidity of $60 \pm 10\%$ with food and water ad libitum. Colon 26 cells (Cell Resource Center for Biomedical Research, Tohoku University) were maintained in RPMI 1640 supplemented 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 atmosphere. A 25-μL volume with 2×10^7 viable tumor cells was inoculated into the right hind footpad of each mouse. The tumor volume was estimated according to a formula that has been described previously (7). Tissue slices of the removed tumor masses were made, and the tumor tissue was confirmed histopathologically.

Experimental design

To assess the temporal expression profile of TfR1 in tumor cells, tumor masses were removed from individual tumor-bearing mice at six different time points (9:00 a.m., 1:00 p.m., 5:00 p.m., 9:00 p.m., 1:00 a.m., and 5:00 a.m.) 7 days after the implantation of tumor cells. The levels of TfR1 protein and mRNA were measured by Western blotting analysis and quantitative reverse transcription-PCR (RT-PCR), respectively. To investigate how the rhythmic variation in TfR1 expression occurs in tumor cells, the influence of CLOCK/BMAL1 and on the transcriptional activity of the TfR1 gene was assessed using luciferase reporter constructs containing wild-type E-box or mutated E-box of the mouse TfR1 promoter, which was based on previous reports. To elucidate the role of c-MYC in the control of the rhythmic expression of TfR1, endogenous c-MYC in Colon 26 cells was downregulated by small interfering RNA (siRNA). The c-MYC–downregulated cells were treated with 50% FBS for 2 hours to synchronize their circadian clock, and the mRNA levels of TfR1 were assessed at 44, 48, 52, 56, 60, 64, and 68 hours after 50% serum treatment. In the same manner as described above, the protein levels of c-MYC and CLOCK were assessed by Western blotting analysis. To explore the temporal binding of endogenous c-MYC and CLOCK to the E-box in the mouse TfR1 gene, chromatin immunoprecipitation analysis was performed in individual tumor masses at 9:00 a.m. and 9:00 p.m. To investigate the function of the 24-hour oscillation of TfR1 expression, time-dependent changes in Pt internalization into tumor cells were assessed using Tf-coupled liposomes encapsulating oxaliplatin (Tf-NGPE L-OHP). The cultured

Colon 26 cells were treated with 50% FBS as described above and then harvested for RNA extraction at 0, 6, 12, 18, and 24 hours after 50% FBS treatment. Nontreated Colon 26 cells harvested at the same time points were used as the control. At 6 or 18 hours after serum treatment, cells were exposed to Tf-NGPE L-OHP (L-OHP, 0.4 mg/mL) for 3 hours. The Pt content in the DNA was measured using an inductively coupled plasma mass spectrometer (ICP-MS). To explore the dosing time–dependent difference in the internalization of Pt into tumor cells in vivo, tumor-bearing mice were injected with a single dose of Tf-NGPE L-OHP at 9:00 a.m. or 9:00 p.m. Plasma and tumor DNA samples were collected only once from individual mice at 1, 3, and 6 hours after injection. The plasma concentration of Pt and its content in tumor DNA were measured as described above. Then, tumor volumes were measured throughout the duration of the experiment.

RT-PCR analysis

Total RNA was extracted using RNAiso (TaKaRa). The cDNAs of mouse TfR1 (NM011638), TfR2 (NM015799), c-Myc (NM010849), and β-actin (NM007393) were synthesized using PrimeScript Reverse Transcriptase (TaKaRa), and the synthesized cDNAs were amplified using GoTaq Green Master Mix (Promega). The PCR products were run on 2% agarose gels. After staining with ethidium bromide, the gel was photographed using Polaroid-type film. The density of each band was analyzed using NIH image software on a Macintosh computer. To evaluate the quantitative reliability of RT-PCR, kinetic analysis of the amplified products was performed to ensure that signals were derived only from the exponential phase of amplification, as previously described (7, 17). We evaluated the validity of our semiquantitative PCR methods using real-time PCR. cDNA was prepared by reverse transcription of total RNA. Real-time PCR analysis was performed on diluted cDNA samples with SYBR Premix Ex Taq Perfect Real-Time (TaKaRa) using a 7500 Real-time PCR system (Applied Biosystems). In addition, as confirmation of RNA extraction from each tumor cell sample, the expression level of Vegf mRNA was measured (Supplementary Data S1).

Western blotting analysis

Nuclear or cytoplasmic proteins in tumor masses were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology). The protein concentrations were determined using a BCA Protein Assay kit (Pierce Biotechnology). The lysate samples were separated on 6% or 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were reacted with antibodies against TfR1 (Zymed Laboratories), c-MYC, CLOCK, β-actin (Santa Cruz Biotechnology), or RNA pol II (Abcam). The immunocomplexes were further reacted with horseradish peroxidase–conjugated secondary antibodies and visualized using Super Signal Chemiluminescent Substrate (Pierce Biotechnology). The membranes were photographed using Polaroid-type film, and the density of each band was analyzed using NIH image software on a Macintosh computer.

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