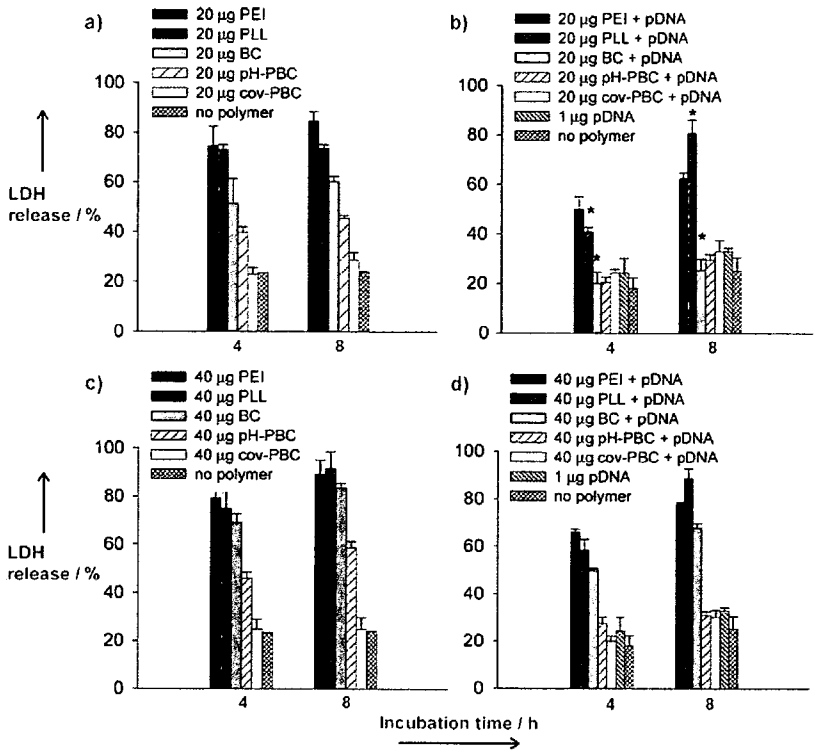

Figure 7. Membrane toxicity of polymers at concentrations of 20 and 40 μg mL^{-1} toward MDA-MB-231 (human breast cancer) cells in DMEM serum-free media. Polymers were incubated both in free form [parts a) and c)] and also pre-complexed to 1 μg pGL3 [parts b) and d)]. Free polymers with cationic components (PEI, PLL, BC) are toxic to cells after just 4 h incubation. However, complexed polymers with concealed PLL (BC, pH-PBC, cov-PBC) effect significantly less LDH release at 20 μg than the static polycations (*$p < 0.05$ using one-way ANOVA). At 40 μg, the presence of excess free polymers in solution with respect to pGL3 (see BC) re-establishes membrane toxicity.

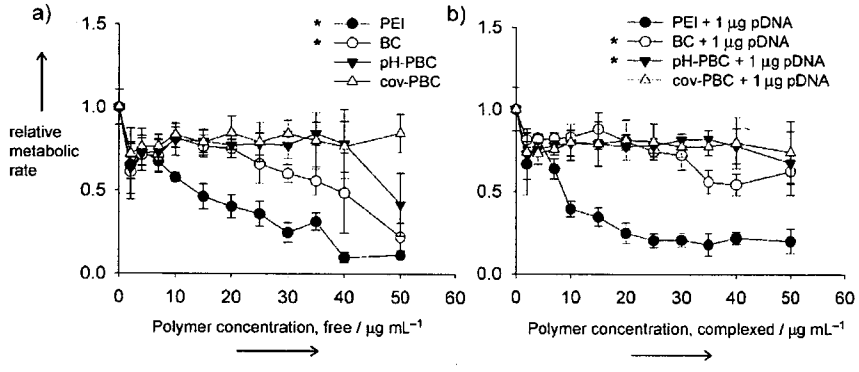

Figure 8. Metabolic toxicity of polymers incubated for 4 h at various concentrations toward MDA-MB-231 (human breast cancer) cells in DMEM serum-containing media. Polymers were incubated in both free form and pre-complexed to 1 μg pGL3. a) Free polymers with cationic components are toxic to cells at higher concentrations, with PEI being more toxic than BC (*$p < 0.05$ using two-way ANOVA). b) When complexed, BC results in negligible toxicity to the cell, similar to pH-PBC particles (*$p > 0.05$ using two-way ANOVA).

Cellular LDH release studies. Polymer interactions with cell membranes were evaluated by monitoring LDH release from MDA-MB-231 human breast cancer cells by using the CytoTox 96 non-radioactive assay from Promega (Madison, WI, USA). One-way ANOVA was used to statistically compare LDH data. MDA-MB-231 cells were seeded in 96-well plates at 25000 cells well^{-1}. After 18 h, the plates were washed with PBS (2×) and serum-free DMEM (90 μL) was added. Each 96-well plate was divided into two sections: maximum LDH and experimental LDH release. Polymer (free or complexed to 1 μg of pGL3) was added to both sections appropriately and incubated with the cells for 4 or 8 h. At the end of each incubation period, PBS was added to the experimental LDH wells (10 μL for volume correction) and 50 μL of the final supernatant was removed and put into a separate 96-well plate. Next, 10 μL of lysis solution (Triton-X, 9% *v/v*) was added to the remaining maximum release wells and incubated for 1 h at 37 °C. Then, 50 μL of the supernatants from these lysed cells were used as controls for determining maximum LDH release. LDH substrate (50 μL) was then added to the separate 96-well plates containing supernatants, covered with aluminum foil, and shaken at room temperature for 30 min before reading the absorbance ($\lambda = 492$ nm).

Cellular metabolic toxicity studies. Metabolic toxicity was assessed through the resazurin dye (Sigma–Aldrich, Milwaukee, WI, USA) by incubating the respective polymers (free or complexed with 1 μg pGL3) in serum-containing media and monitoring the toxic effects of the polymers on the metabolic rates of cells through reduction of the dye. Briefly, 96-well plates were seeded at 20000 cells well^{-1} and incubated for 24 h. Free polymer at increasing concentrations of 0–50 μg mL^{-1} was added to wells and incubated in serum-containing media for 4 h before refreshing the media. After 18 h incubation, 10 μL of resazurin dye (60 μM in PBS) was added to each well and incubated for 4 h before measuring fluorescence ($\lambda_{ex} = 560$ nm, $\lambda_{em} = 590$ nm). Cell viability is reported as relative metabolic rate with respect to controls without polymers. Two-way ANOVA was used to compare the data.

Cell transfection studies. MDA-MB-231 (human breast cancer), C2C12 (murine myoblast) and MCF-7 (human breast cancer) cell lines were obtained and cultured according to ATCC protocols. COS-7 cells (Green African monkey kidney) were obtained from David M. Lynn (University of Wisconsin). 4T-1 cells (murine colon cancer) were obtained from the Small Molecule Screening Facility

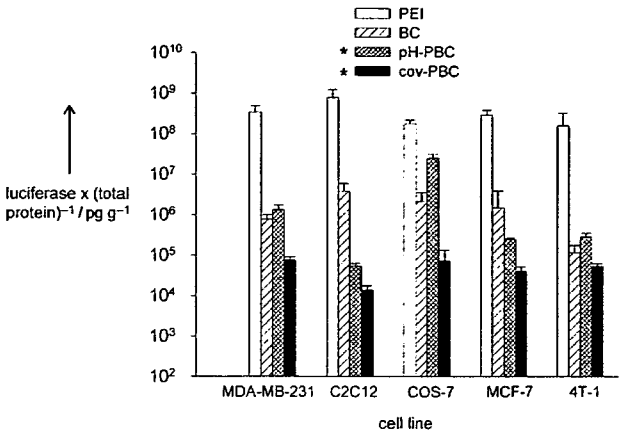

Figure 9. Transfection efficiency of the polymers in MDA-MB-231 (human breast cancer), C2C12 (murine myoblast), COS-7 (Green African monkey kidney), MCF-7 (human breast cancer) and 4T-1 (murine colon cancer) cells when incubated in the respective serum-containing media for 4 h. All polymers were formed at N/P = 20 (optimal ratio determined in tests with N/P = 7–50) with 2 μg pGL3. pH-PBC and BC PICs consistently effect significantly higher gene expression relative to cov-PBC across all cell lines (*$p < 0.05$ using one-way ANOVA in each case).

Center (University of Wisconsin) and cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. The luciferase assay system was purchased from Promega. Luminescence was measured with a microplate Tropix luminometer (Applied Biosystems). Protein content was obtained using the DC protein assay kit from BioRad (Hercules, CA, USA), absorbance was measured with the Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA), and results were fit to a known protein calibration curve. Cells were seeded at either 300000 cells well^{-1} in 6-well plates or 150000 cells well^{-1} in 12-well plates and cultured for 24 h. The next day, wells were aspirated, washed with PBS (2 × 1 mL), and appropriate medium for each cell line was added (supplemented with 10% FBS and 1% penicillin/streptomycin). Complexes (2 μg pGL3 well^{-1}) formed at the indicated N/P ratio (N/P = 1 is defined as the minimum amount of polymer required to retard 1 μg of pGL3 on a 0.75% agarose electrophoretic gel) were added to wells 4 h before refreshing the media. After 12 h, plates were assayed with the Promega luciferase assay system. Luciferase relative light units (RLUs) were converted into units of concentration by using known luciferase concentration standards. One-way ANOVA was used to compare transfection data. A value of $p < 0.05$ was considered statistically significant.

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Keywords: block copolymers · gene delivery · intracellular trafficking · nanoparticles · polymers

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Supramolecular nanocarriers integrated with dendrimers encapsulating photosensitizers for effective photodynamic therapy and photochemical gene delivery†

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Recently, biomedical applications of dendrimers, three-dimensional tree-like macromolecules, have received much attention. In this paper, we introduce new biomedical applications of functional dendrimers: dendrimers encapsulating photosensitizers composed of a center dye molecule surrounded by poly(benzyl ether) dendrons with ionic peripheral groups as a new type of photosensitizer (PS) for photodynamic therapy (PDT). Dendrimers encapsulating porphyrin and with ionic peripheral groups (DPs) spontaneously formed polyion complex (PIC) micelles through electrostatic interactions with oppositely-charged block copolymers, and showed no self-quenching of the center dye molecule inside the micellar core due to a unique DP structure, leading to remarkable *in vitro* photocytotoxicity. The DP-incorporated micelles showed successful treatment of choroidal neovascularization (CNV) in rats without any sign of side effects. Thus, the DP-incorporated micelles are expected to be an innovative PS formulation for successful PDT against ophthalmic diseases. In this paper, we also review polymeric micelles incorporating phthalocyanine core dendrimers (DPc) for cancer PDT and novel light-responsive supramolecular gene carriers integrated with DPc for site-directed transfection *in vivo*.

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Introduction

Photodynamic therapy (PDT) has been attracting increasing attention as a promising method for the treatment of solid tumors and ophthalmic diseases.[1-3] PDT involves administration of photosensitizers (PSs) to the body, followed by activation of PSs at the diseased site using light of specific wavelength. Upon photoirradiation, PSs undergo photosensitized reaction processes of type I and type II. The former produces free radicals or superoxide anions resulting from hydrogen- or electron transfer from the photoexcited PSs,

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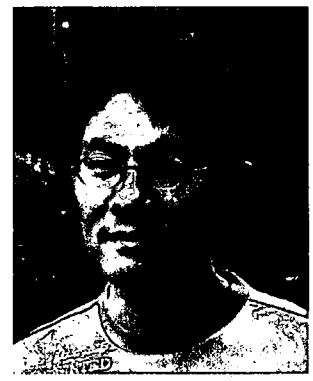

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whereas the latter generates cytotoxic singlet oxygen ($^1O_2$) by the energy transfer. In general, the type II reaction is known to be more efficient in PDT.

A challenge in PDT is to create efficient PSs, which show highly selective photocytotoxicity to the diseased tissues, and an increasing number of PSs are presently being explored in preclinical and clinical studies.[1,2] PSs generally have large π-conjugation domains, such as a porphyrin structure, to obtain a high quantum yield and effective energy absorption. Therefore, most conventional PSs easily form aggregates in aqueous media through their π–π stacking and hydrophobic interactions, resulting in the self-quenching of the excited state. This may decrease the photodynamic effect of the PS after its accumulation in the diseased tissue. On the other hand, there is also a strong incentive to develop effective drug delivery systems (DDS) for PSs, to enhance the selectivity and effectiveness of PDT as well as prevent side effects caused by non-specific distribution of PSs in the body, such as prolonged skin hypersensitivity to sunlight.[2] To date, a variety of DDS, including polymer–PS conjugates,[4] long-circulating liposomes[5] and polymeric micelles,[6] have been examined; however, the aforementioned properties of PSs, *i.e.*, a strong tendency to form aggregates and the related self-quenching effect, might interrupt the development of effective PS formulations. In general, it might be difficult to effectively incorporate such hydrophobic substances into drug carriers. Also, increased loading of PSs into drug carriers could change the modality of photochemical reaction from type II to type I due to aggregate formation, leading to diminished photodynamic efficacy.[7] Thus, both efficiencies of the PS delivery and photochemical reactions of PS itself should be considered in the development of DDS in PDT.

Recently, we have developed a new type of PS based on a dendritic architecture, *i.e.*, dendrimers encapsulating porphyrin with ionic peripheral groups (DPs), in which the centre porphyrin is surrounded by the 3rd generation of poly(benzyl ether) dendrons (Fig. 1).[8] Unlike conventional PSs, DPs ensure a high efficacy of singlet oxygen production even at a high concentration, since the dendritic envelopes of DPs can prevent aggregation of the center porphyrin (Fig. 2). Thus, it is assumed that the encapsulation of DPs into drug carriers might not compromise the singlet oxygen production efficiency. In addition, the peripheries of DPs can be modified with a variety of functional groups, providing water-solubility and practical functions such as tissue-targetability. Also, the introduction of charged ionic groups into the periphery of a DP allows its stable incorporation into a supramolecular nanocarrier, a polyion complex (PIC) micelle, through electrostatic interactions with oppositely-charged block copolymers as schematically shown in Fig. 2.

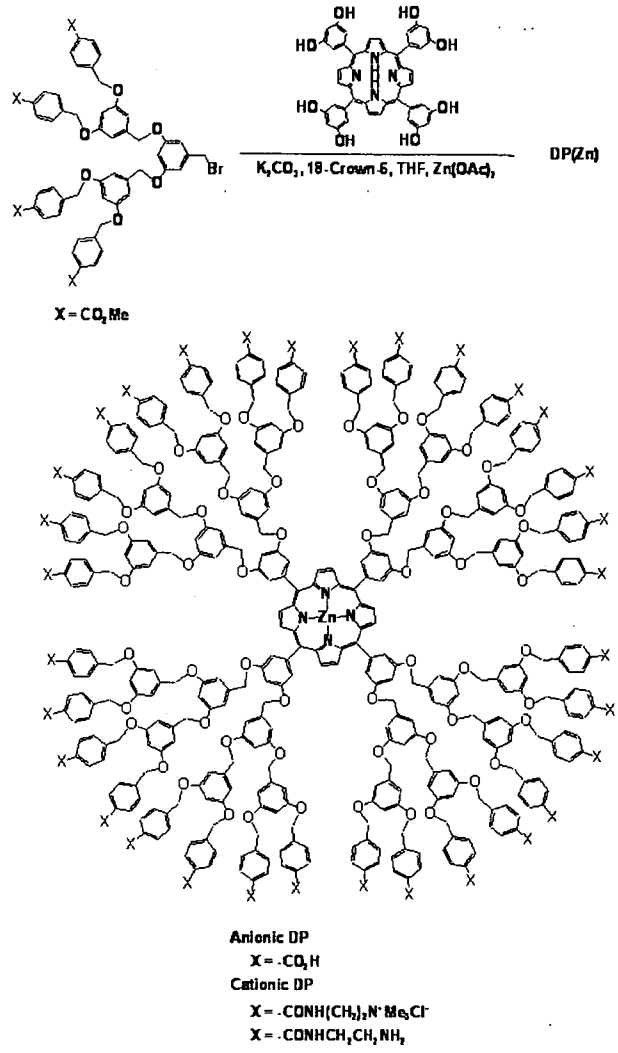

Fig. 1 Chemical structures of dendrimers encapsulating porphyrin with ionic peripheral groups (DPs) and the synthetic scheme.

Polymeric micelles, self-assemblies of block copolymers, are characterized by a size of several tens of nanometres, a fairly narrow size distribution, remarkably low critical micelle concentration (c.m.c.) and segregated core–shell structure, and they have recently received considerable attention as a promising modality of drug carriers.[9–11] It has been demonstrated that polymeric micelles could circulate stably in a bloodstream and, therefore, accumulate effectively in solid tumors[12] due to the enhanced permeability and retention (EPR) effect, characterized by microvascular hyperpermeability and impaired lymphatic drainage in tumor tissues.[13] Also, we have

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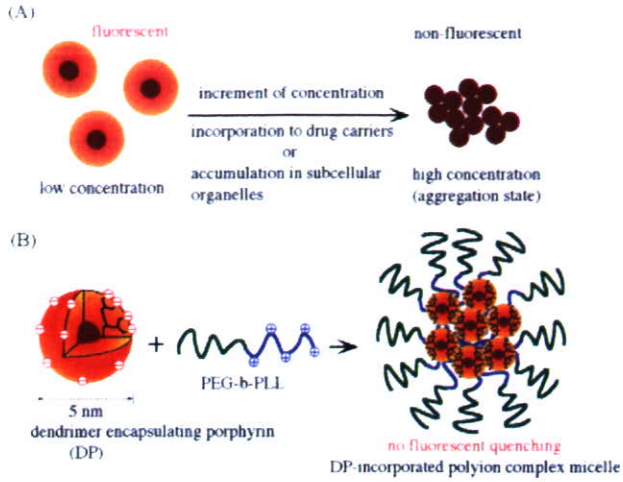

Fig. 2 (A) Conventional PSs form aggregates at a high concentration, resulting in their self-quenching. (B) Formation of DP-incorporated polyion complex (PIC) micelles through electrostatic interactions between anionic DPs and poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-*b*-PLL) copolymers. The dendritic structure of DP can sterically prevent aggregation of the center porphyrin, thus there is no fluorescent quenching of the center porphyrin. [Ref. 12, copyright permission from Wiley-VCH].

recently reported that polymeric micelles also accumulated in the choroidal neovascularization (CNV) site in rat models,[14] suggesting the targeted therapy of ophthalmic diseases by polymeric micelles. The PIC micelle, a new type of polymeric micelle formed through the electrostatic interaction between a pair of charged block copolymers and oppositely-charged macromolecules,[15] is expected to be a targetable carrier system for biologically active molecules, including proteins[16] and nucleic acids[17,18] as well as ionic DPs.[19–22]

In this paper, we review recent advances in the field of PIC micelles incorporating photosensitizer core dendrimers for effective PDT. Also, we introduce our new technology for the control of transgene expression by using gene nanocarriers integrated with dendrimers encapsulating photosensitizers.

Dendrimers encapsulating porphyrins (DPs) as a new photosensitizer

Synthesis of ionic DPs

There are two different synthetic strategies towards ionic DPs; a divergent and a convergent growth approach are generally employed to construct dendrimer frameworks.[23] DPs have poly(benzyl ether) dendritic wedges, which were first synthesized by Fréchet and Hawker using the convergent approach.[24] Aida *et al.* reported the first example of a dendrimer encapsulating a metalloporphyrin, in which the zinc porphyrin functionality is covalently encapsulated by poly(benzyl ether) dendritic wedges with methoxycarbonyl groups on the exterior surface, on the basis of Fréchet and Hawker's convergent approach.[25] Fig. 1 shows the synthetic scheme towards DPs. Alkaline-mediated coupling of 5,10,15,20-tetrakis(3′,5′-dihydroxyphenyl)porphine with methoxycarbonyl-terminated aryl ether dendritic bromides gave a DP, which was metalated with $Zn(OAc)_2$, followed by hydrolysis of the exterior MeO_2C-groups with KOH. Dialysis of the reaction mixture for 2 days in fresh water to remove excess KOH, followed by evaporation to dryness, gave an anionic DP bearing 32 carboxylic groups on the periphery. Also, the synthesis of a cationic DP, bearing 32 quaternary ammonium groups on the periphery, was performed by amidation of the anionic DP with 1-amino-2-(trimethylammonium)ethane, followed by purification by column chromatography and preparative HPLC using THF as an eluent. Likewise, a DP bearing 32 primary amino groups was prepared by amidation of the anionic DP with *N*-(trifluoroacetyl)ethylene-1,2-diamine, followed by quantitative deprotection of the trifluoroacetyl group. The perfection of cationic DPs after amidation of anionic DP was confirmed by MALDI-TOF-MS.[21]

Properties of ionic DPs as a photosensitizer for PDT

As described in the Introduction, DPs show a unique photochemical property that the center porphyrin might not be quenched even at a high concentration due to its spatial isolation in the focal core of the dendrimer. Thus, the photochemical reaction of DPs may not be affected by their subcellular localization and local concentrations. Regarding the photochemical properties, ionic DPs absorb light at 415, 434 (Soret bands) and 559 nm (Q-band) and emit fluorescent light at 610 and 660 nm. In our previous study, the singlet oxygen quantum yield of DPs was measured by direct observation of the O_2 ($^1\Delta_g$) → O_2 ($^3\Sigma_g^-$) transition at 1270 nm ((0,0) vibronic band) in MeOD.[8] It was demonstrated that ionic DPs showed similar singlet oxygen quantum yield to a conventional PS, protoporphyrin IX. Thus, the center porphyrin possesses a high singlet oxygen quantum yield in spite of its dendritic structure. In the photodynamic action of DPs, only the singlet oxygen or other reactive oxygenic species (ROS) is assumed to play an essential role, because the direct interaction of dye radicals with other compounds should be minimal in the dendritic architecture. Also, DPs may be hardly photobleached during photochemical reactions, although this effect remains to be elucidated. It is generally known that singlet oxygen has a quite short lifetime in an aqueous milieu, and the diffusion distance of $^1O_2$ is estimated to be less than 10 nm.[2] Nevertheless, the 3-D structure of dendrimers with the size of 5 nm appeared not to be a barrier for the $^1O_2$-induced photodynamic effect, because ionic DPs showed comparable or even more efficient photocytotoxicity compared with conventional PSs.[8] Possibly, the lifetime of $^1O_2$ may be prolonged inside the hydrophobic dendritic structure of DPs.

DPs are hydrophilic macromolecules (~5 nm), so that they are assumed to be taken up by the cells through endocytic pathways. Nevertheless, the ionic DPs showed different cellular associations depending on the peripheral charged groups. In our previous study, cationic DP showed rapid association to the negatively-charged plasma membrane through electrostatic interaction, followed by internalization by adsorptive endocytosis, whereas anionic DPs were slowly internalized by fluid phase endocytosis due to the electrostatic repulsion.[8] Thus, both cationic and anionic DPs finally localized in

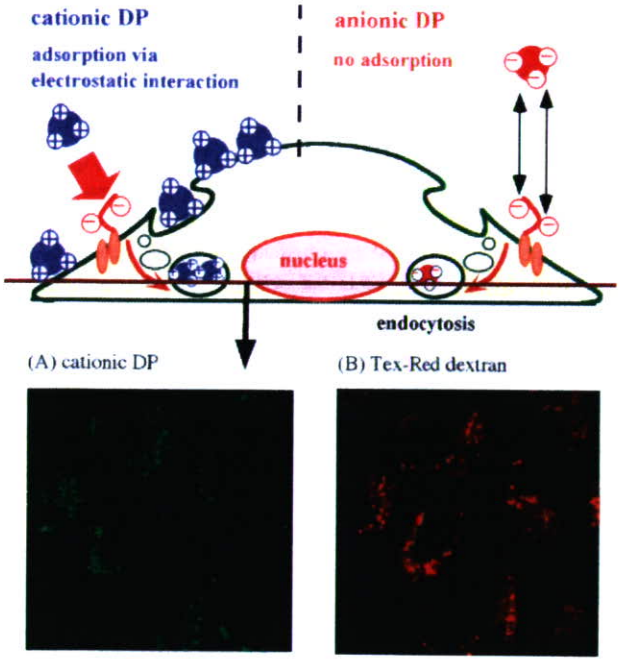

Fig. 3 Schematic illustration of the cellular association manners of cationic and anionic DPs, and confocal images of cationic DP (A) and Tex-Red dextran, an endocytosis marker (B) in LLC cells after incubation for 3 h at 37 °C. [Ref. 8, copyright permission from American Chemical Society].

endosomal compartments, as demonstrated by colocalization with Tex-Red dextran, a fluid phase endocytic marker (Fig. 3). Although cationic DP showed only 22–25 times higher cellular association than anionic DP, the photocytotoxicity ($^1O_2$-induced cytotoxicity) of cationic DP was 230 times higher than that of anionic DP.[8] Such a difference in the photocytotoxicity between cationic and anionic DPs may be due to different interactions of both compounds with cellular components. That is, cationic DP is likely to electrostatically interact with negatively-charged cell membranes, photodamaging membrane components, which may be susceptible to $^1O_2$-induced cell death.

Fluorescent microscopy observation using organelle-specific dyes revealed that the photodamage by DPs maintained the characteristic structure of cell membranes and intracellular organelles (the plasma membrane, mitochondrion and lysosome), whereas such organelles were severely photodisrupted by conventional PSs.[8] Nevertheless, cationic DP induced efficient cell death. In this regard, the fluorescence of Rhodamine 123 (Rh123), a mitochondrion marker, was significantly attenuated by the photodamage by cationic DP. The fluorescence intensity of Rh123 is known to be correlated with the amount of adenosine triphosphate (ATP) in the cell.[26] Therefore, cationic DP may have clipped ATP production in the cell, leading to efficient cell death. The eliminated ATP production may be caused by exhaustion of intracellular oxygen or photodamage to the plasma membrane.[27,28] Also, there is another possibility that the photodamage to the cell membranes could disrupt the endosome/lysosome, localizing DPs in cytoplasmic organelles, which are susceptible to $^1O_2$-induced cell death.[29] For example, it was reported that the photodamage to the mitochondrion induced apoptosis through the cytoplasmic release of cytochrome c.[30] The exact molecular target of ionic DPs is an issue to be clarified in the future.

Low dark toxicity of PS is one of the important criteria for assessing the usefulness of PS because unwanted toxicity to normal tissues is one of major side effects in clinical PDT.[31] Ionic DPs showed extremely low dark toxicity even after prolonged incubation of 72 h.[8] The extremely low dark toxicity of DPs may result from the distinctive intracellular disposition characteristics due to their relative large size (~5 nm).

DP-incorporated polyion complex (PIC) micelles for effective PDT

Physicochemical properties of DP-incorporated PIC micelles

The DP-incorporated PIC micelles are formed from a pair of charged block copolymers of poly(ethylene glycol)-*block*-poly(L-lysine) (PEG-*b*-PLL) or poly(ethylene glycol)-*block*-poly(aspartic acid) (PEG-*b*-PAA) and oppositely-charged DPs with 32 charged groups on the periphery (Fig. 2B). Simple mixing of block ionomers and ionic DPs at a stoichiometric charge ratio led to the spontaneous formation of the PIC micelles. The PIC micelles prepared from negatively-charged DP and positively-charged PEG-*b*-PLL had a diameter of approximately 64 nm with an extremely narrow size distribution in physiological saline solution. The spherical shape of the DP-incorporated micelles was confirmed by observation with atomic force microscopy (AFM) and field emission-transmission electron microscopy (FE-TEM). The static light scattering (SLS) measurement revealed that an individual PIC micelle contains an average of 38 DP molecules.[19] From the standpoint of the application as drug carriers, the stability of the PIC micelles against salt concentrations might be primarily important, since the electrostatic shielding of polyelectrolytes with salt ions might destablize the PIC structure.[32] In this regard, the PIC micelles consisting of cationic DP bearing 32 quaternary ammonium groups–PEG-*b*-PAA showed a higher stability against NaCl concentrations than those of PLL_{27}–PEG-*b*-PAA.[19] This may be explained by the assumption that the PIC from a rigid dendrimer may produce a smaller entropy gain upon dissociation than that from a flexible PLL homopolymer. In the case of the PIC micelles consisting of cationic DP bearing 32 primary amino groups–PEG-*b*-PAA or anionic DP–PEG-*b*-PLL, the complexation between DPs and block copolymers might be accomplished by the formation of hydrogen bonds between carboxylic acid and primary amine groups after proton transfer from the acid amine. Therefore, the resulting polyion complex micelles showed a remarkable stability against an increase in NaCl concentrations up to 1500 mM, 10 times higher than the physiological concentration.[19,21]

The PIC micelles of anionic DP–PEG-*b*-PLL showed pH-dependent structural changes.[22] In this study, the translational diffusion coefficient (D_T) and normalized $(Kc/\Delta R(0))^{-1}$ (normalized to the micelle at pH 7.4) of the PIC micelles, where D_T is related to the hydrodynamic diameter based on

the Stokes–Einstein equation, and the normalized $(Kc/\Delta R(0))^{-1}$ value is related to the changes in the apparent molecular weight of the micelles, were measured under different pH conditions by dynamic light scattering (DLS) and SLS measurements, respectively. Both the hydrodynamic diameter and normalized $(Kc/\Delta R(0))^{-1}$ value remained constant in the pH range from 6.4 to 8.5. However, when the pH was below 6.4, the PIC micelles showed a gradual increase in the diameter and apparent molecular weight, and finally underwent precipitation at pH 5.6, indicating the acid-responsive feature of the micelles. Under acidic pH conditions, the carboxylic acid groups of DP might be considerably protonated, leading to diminution of the electrostatic interaction between anionic DP and PEG-*b*-PLL, thus the well-defined core–shell structure may become more obscure and a merging of the micelles may take place. The similar pH-dependent structural changes of the PIC micelles were also observed for the system of cationic DP bearing 32 primary amino groups–PEG-*b*-PAA.[21] Such a pH-responsive behavior of the micelles allows their effective accumulation in solid tumors in response to a low pH condition in the tumor tissue (~pH 6.5)[33] or an intracellular acidic endosomal compartment (~pH 5.0) while their stable circulation in the bloodstream is expected.

In vitro photodynamic effect of DP-incorporated PIC micelles

In the UV-Vis absorption spectra, the PIC micelles of anionic DP–PEG-*b*-PLL showed a red shift of 5 nm for the Soret band of the porphyrin core and a hypochromicity of about 5%,[22] which seems to be consistent with the previous reports on the electrostatic assembly of charged porphyrins and oppositely-charged compounds.[34,35] The cancellation of the charge repulsion of the negatively-charged DP surface by the formation of the electrostatic assembly may lead to shrinkage of the hydrophobic dendrimer frameworks, which may be related to the hypochromicity.[25] Importantly, the PIC micelles showed no fluorescent quenching of DP (630 nm), although the local concentration of DP is assumed to be extremely high in the core of the PIC micelles.[22] Similarly, no fluorescent quenching of DP in the PIC micelles was observed for the system of cationic DP bearing 32 primary amine groups–PEG-*b*-PAA.[21] It is worth noting that no fluorescent quenching of DP was microscopically observed inside the cell incubated with the DP-incorporated PIC micelles,[22] suggesting the effective photochemical reaction in the living cell directed the enhanced photocytotoxicity. It is hypothesized that the dendritic envelope of DP might prevent the collisional quenching of the center porphyrin even at an extremely high concentration. This unique photochemical property of the DP-incorporated PIC micelles might not be achieved by other conventional PSs and PS formulations.

In regard to the efficiency of the photochemical reactions, the DP-incorporated PIC micelles showed an oxygen consumption rate comparable to free DP in phosphate-buffered saline containing fetal bovine serum (FBS) as a singlet oxygen acceptor (Fig. 4).[22] This result suggests that the singlet oxygen molecules produced from DP can escape from the micellar structure and react with proteins of FBS. Thus, the DP-incorporated PIC micelles might have the capacity to efficiently photo-oxidize biomolecules in the solution without their structural changes. Possibly, the DP-incorporated PIC micelles may achieve the elevated concentration of local singlet oxygen, which may not be obtained by other formulations containing conventional PSs.

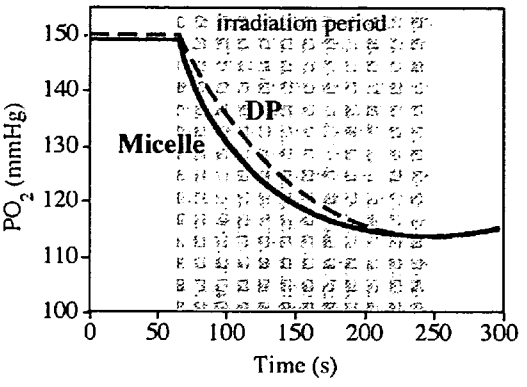

Fig. 4 Profiles of the oxygen consumption by anionic DP (--) and the DP-incorporated micelles (—) in phosphate-buffered saline solution containing 10% fetal bovine serum (FBS). The light irradiation and the oxygen partial pressure measurement were performed using an Hg lamp and a Clark-type oxygen microelectrode, respectively. [Ref. 12, copyright permission from Wiley-VCH].

Very interestingly, the PIC micelles of anionic DP–PEG-*b*-PLL showed approximately 280 times more efficient photocytotoxicity (against Lewis lung carcinoma (LLC) cells) compared with free anionic DP (Fig. 5), although incorporation of DP into the PIC micelles resulted in an only 6–8 times increase in the cellular uptake.[22] From a practical standpoint, such a manner of the photocytotoxicity enhanced by the micelle formulation may avoid long-term phototoxicity after PDT, since the DP-incorporated micelles are assumed to gradually dissociate to constituent DP and block copolymers in the body. It may be hypothesized that the DP-incorporated

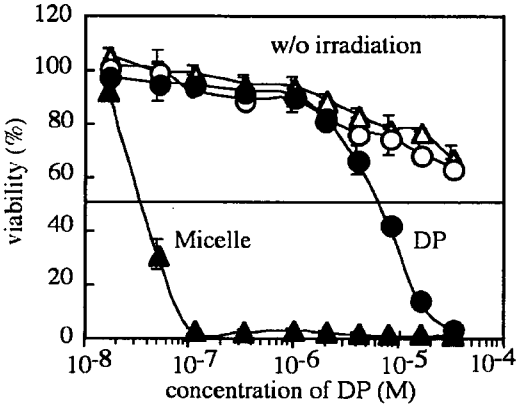

Fig. 5 The photocytotoxicity (closed symbols) and the dark toxicity (open symbols) of anionic DP (circles) and the DP-incorporated micelles (triangles) against LLC cells. In this assay, the cells were photoirradiated for 10 min with broadband visible light using a xenon lamp (150 W) equipped with a filter passing light of 400 700 nm (fluence: 180 kJ cm^{-2}). The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

micelles may have a specific mechanism to enhance their photocytotoxicity. Recently, PEGylated chlorin-e_6 as well as PEG-based polymeric micelles were reported to show enhanced localization in several cytoplasmic organelles, including the mitochondrion.[30,36] Thus, the PEG shell of the PIC micelles may have a role in altering the intracellular mechanism of DP to increase the photocytotoxicity. Otherwise, the photodamage-induced disruption of the endosomal/lysosomal membranes may assist the localization of DP in the cytoplasmic organelles, which may be susceptible to $^1O_2$-induced cell death. In any case, the remarkably increased concentration of local singlet oxygen by encapsulation into the PIC micelles is likely to contribute significantly to the enhanced photocytotoxicity of DP. Further investigations to address the detailed mechanisms of the enhanced photocytotoxicity of the DP-incorporated micelles are now in progress in our research group.

PDT using DP-incorporated PIC micelles

The exclusive age-related macular degeneration (AMD), a condition caused by choroidal neovascularization (CNV), is a leading cause of visual loss in developed countries.[3] Recently, Visudyne®, a liposomal formulation of verteporfin, has been demonstrated to be effective for the treatment of AMD, and has been approved for clinical use.[37] However, PDT with Visudyne requires repeated treatments every three months, and many patients nevertheless suffer from visual loss due to recurrence of CNV. Therefore, there is a strong incentive to develop more effective formulations of PSs for the PDT treatment against AMD.

Recently, we applied the PIC micelles of anionic DP–PEG-*b*-PLL for PDT of experimental CNV in rats, which was created by laser photocoagulation.[38] When DP-incorporated micelles were intravenously administered, they showed effective and selective accumulation in the CNV lesions.[38] Laser irradiation (5–50 J cm^{-2}) at 4 h after iv administration of DP-incorporated micelles resulted in successful occlusion of CNV in 60–72% of the tested animals. Importantly, approximately 80% of the tested animals showed maintained CNV occlusion even 7 days after laser irradiation.[38] In contrast, it was reported that PDT with Visudyne under the same experimental conditions showed less effectiveness in CNV occlusion and resulted in recurrence of CNV 7 days after the treatment.[39] Thus, DP-incorporated micelles might be an effective PS formulation, and may not require the repeated treatments, which are problems of other PS formulations. In this study, the skin photodamage was also evaluated by photoirradiating the mouse abdominal skin with broadband visible light (Xenon lamp equipped with a filter passing light of 377–700 nm) 4 h after iv injection of DP-incorporated micelles or Photofrin, a clinically used PS formulation for cancer therapy. As a result, mice treated with DP-incorporated micelles did not experience macroscopically observable skin damage, whereas those treated with Photofrin displayed severe skin damage.[38] Thus, DP-incorporated micelles might circumvent skin hyperphotosensitivity, a major side effect of current PDT, due to their reduced accumulation in the skin. From these *in vivo* results, we can conclude that DP-incorporated micelles may have an innovative PS formulation for the treatment of ophthalmologic diseases.

Dendrimer encapsulating phthalocyanine as a photosensitizer for cancer therapy

We have demonstrated that DP-incorporated micelles are a potent PS formulation for PDT against ophthalmologic diseases. However, DPs have relatively short excitation wavelengths (*i.e.*, 430 and 560 nm), which might be a limitation for PDT except for the transparent tissues such as ophthalmologic organs. Skin tissue has melanin dyes, which absorb short wavelength light to prevent photochemical genetic disorder, and also heme proteins of red blood cells account for most of the light absorption in the visible region. Such light absorption by human bodies prevents the excitation of PSs for photochemical reactions. Therefore, PSs should have long absorption wavelength for the PDT of deeper lesions such as solid tumors.

In this regard, several phthalocyanine molecules have been widely studied as potential PSs with an appropriate absorption wavelength for practical PDT application.[2,40] The phthalocyanines have an optical absorption at approximately 680 nm, where the light can penetrate tissues 2 times deeper than Photofrin (630 nm). Recently, we have synthesized dendrimers encapsulating phthalocyanine with ionic peripheral groups (DPc, Fig. 6) with the maximum absorption at 685 nm according to the synthetic procedure established by Ng *et al.*,[41] and prepared DPc-incorporated micelles, having the size of approximately 50 nm, through the electrostatic interaction between anionic DPc and PEG-*b*-PLL.[42] DPc-incorporated micelles were stable in a phosphate-buffered solution containing 10% FBS, maintaining the size and polydispersity of the micelles. The micelle formation was accompanied by a shift of maximum absorption wavelength of DPc from 685 nm

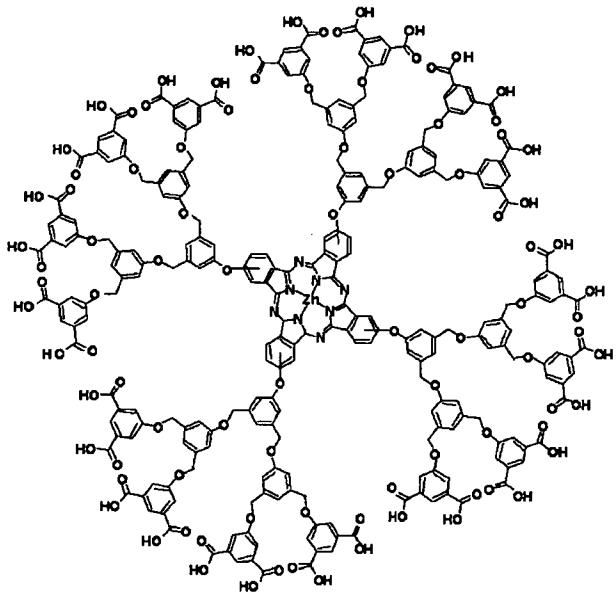

Fig. 6 Chemical structure of dendrimer encapsulating phthalocyanine (DPc).

to 630 nm, indicating some interactions between the phthalocyanine units of DPc (*i.e.*, aggregate formation) in the micellar core. The relatively small dendritic wedges may be insufficient to prevent such interactions. As a result, DPc-incorporated micelles showed reduced oxygen consumption rates compared with DPc alone.[42] Nevertheless, DPc-incorporated micelles showed significant enhancement of the light-induced cytotoxicity, which greatly relied on the photoirradiation time. In contrast, free DPc did not exhibit such enhancement of the photocytotoxicity regardless of irradiation time. Thus, DPc-incorporated micelles achieved approximately 100-fold photocytotoxicity compared with free DPc after 60 min photoirradiation. The treatment of solid tumors by PDT with DPc-incorporated micelles is ongoing in our laboratory, and the results will be reported elsewhere in the near future.

Photochemical gene delivery using dendrimer encapsulating phthalocyanine (DPc)

The DPc-incorporated micelles are taken up by the cells through the endocytic pathway, preferentially localizing in the endosome or lysosome. Upon photoirradiation, DPc-incorporated micelles may photochemically disrupt the endosomal/lysosomal membrane to relocalize in the subcellular organelles susceptible to $^1O_2$-induced cell death. This process is available for the light-induced cytoplasmic delivery of macromolecular compounds such as plasmid DNA (pDNA), which are impermeable to cell membranes and easily digested in the endosome or lysosome. This concept was "photochemical internalization (PCI)" by Berg and Høgset *et al.*[29,43 46] It should be noted that the light dose necessary for PCI is much lower than that for PDT, ensuring the low photocytotoxicity of PCI. Recently, we also carried out *in vitro* PIC-mediated gene transfection using a combination of the DPc–PEG-*b*-PLL and pDNA–PEG-*b*-PLL micelles, and achieved 100-fold photochemical enhancement of the transgene expression while maintaining 80% cell viability over a wide range of DPc concentrations and light doses.[47] Moreover, we optimized the chemical structures of PEG-*b*-polycations forming the pDNA-incorporated micelles for the PCI-mediated transfection, and found that the use of PEG-*b*-polycations having the repeated ethylenediamine units in the side chain allowed approximately 1000-fold light-selective gene transfection,[48] suggesting that the PCI and the so-called proton sponge effect[49] may work synergistically to enhance the transfection efficiency.

From the standpoint of *in vivo* applications of the PCI-mediated transfection, gene carriers should be equipped with a photosensitizing unit as one component, motivating us to develop the ternary complexes composed of pDNA, quadruplicated cationic peptide (CP_4) containing nuclear localization signal (NLS) sequence and anionic DPc (Fig. 7).[50] The simple addition of anionic DPc to the cationic pDNA–CP_4 complexes led to the spontaneous formation of the ternary complexes having the pDNA–CP_4 core surrounded by the DPc envelope, and the obtained nanoparticles had the size of 130 nm with a narrow distribution. Interestingly, such nanoparticles were not obtained by the addition of poly(aspartic acid) homopolymer with a polymerization degree of 26 to the pDNA–CP_4 complexes, suggesting that the 3-D structure of DPc might play an essential role in the formation of the ternary complexes. Also, such ternary complexes are definitely discriminated from the layer-by-layer assemblies,[51,52] because a core of hard materials is not required for the formation of the ternary complexes. The pDNA–CP_4–DPc ternary complexes are assumed to activate the transgene expression in a light-inducible manner according to the following processes: (1) internalization of the ternary complexes through endocytosis, (2) release of DPc from the ternary complexes due to protonation of the peripheral carboxyl groups of DPc under acidic conditions in the endosome, (3) interaction with the endosomal membrane due to the hydrophobic nature of the dendrimer framework, (4) light-induced endosomal escape of the pDNA–CP_4 complexes through the photochemical disruption of the endosomal membrane, and (5) nuclear transport of the pDNA–CP_4 complexes guided by the NLS sequence.[53] As a result, the ternary complexes showed a more than 100-fold light-induced enhancement of *in vitro* transgene expression while maintaining the cell viability.[50] The PCI-mediated transfection with the ternary complexes was not accompanied by long-term toxicity, which was observed by transfection using polyethylenimine (PEI), a well-known highly transfectable polycation. Thus, the ternary complexes might avoid the cytotoxicity induced by buffering polycations, because the cytotoxic events might occur only during the photoirradiation. To demonstrate the potential of the ternary complexes for *in vivo* gene transfer, the ternary complexes were subjected to subconjunctival injection in rats, followed by laser irradiation with a semiconductor laser (689 nm) at 2 h post-injection (Fig. 8). The PCI-mediated gene transfer with the ternary complexes resulted in appreciable gene expression of the fluorescent protein (*Venus*) only at the laser-irradiated site in the conjunctiva.[50] In contrast, the subconjunctival injection of the pDNA–PEI complexes failed

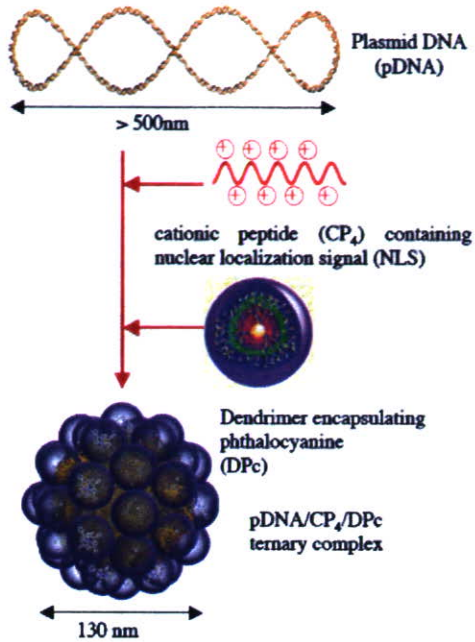

Fig. 7 Formation of the pDNA CP_4 DPc ternary complexes as light-responsive gene carriers for site-directed gene transfer.

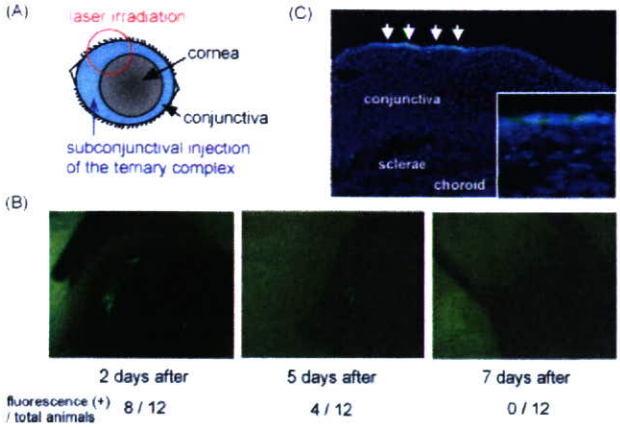

Fig. 8 Light-induced gene transfer to the rat conjunctival tissue. (A) Scheme for *in vivo* transfection. Rats were given a subconjunctival injection (colored in light blue) of the ternary complexes containing pDNA encoding a fluorescent protein, *Venus*. At 2 h post-injection, a part of the conjunctiva (red circle) was irradiated by the laser. (B) Fluorescent images of *Venus* expression in the rat eye at 2, 5 and 7 days after the transfection. The rates of the fluorescent-positive eyes (fluorescent (+)/total eyes) are indicated below the images. (C) Fluorescent image of the *Venus* expression at 2 days after the transfection in the frozen section of the conjunctival tissue. The cell nuclei were stained in blue. [Ref. 50.]

in the gene transfection. Thus, we succeeded in the control of the *in vivo* transgene expression by using light-responsive gene carriers integrated with DPs.

Future prospects

In this paper, we have reviewed the characteristics of the DP-incorporated PIC micelles from the standpoint of the photochemical reactions and *in vitro* and *in vivo* photodynamic effect. Our results revealed that the DP-incorporated micelles possess unique photochemical properties and show remarkable *in vitro* photodynamic effect, which have not been achieved by other PS formulations. The PDT with DP-incorporated micelles succeeded in the treatment of rat AMD models, without any sign of side effects, facilitating the development of the DPc-incorporated micelles for cancer photodynamic therapy. Also, the PCI-mediated transfection with gene carriers integrated with DPc allowed the control of transgene expression in the body using light irradiation as an external stimulus. Importantly, the PCI-mediated transfection may not require as high an irradiation energy as PDT, suggesting its potential use for the treatment of deeper tissues, to which PDT is not applicable.

The recent advance in laser technology will allow diverse clinical applications of PDT. For example, the two-photon excitation by using near infrared lasers may have great potential for the PDT of diseases in deeper tissue (*e.g.*, brain tumors). The possibility of two-photon absorption is known to depend linearly on the intrinsic two-photon cross section of the compounds, δ, and quadratically on the incident light intensity.[54] Although the two-photon cross section of molecules is typically small compared with one-photon absorption, neodymium:YAG (Nd:YAG) laser emitting at 1064 nm or tunable solid state lasers generating far-red/near infra-red light could significantly increase the possibility of two-photon absorption of the compounds due to high laser output.[55] In the light of such situations, the development of the delivery systems to improve the biodistribution and enhance the photodynamic efficacy of PSs will be a key technology to effective PDT. Our nanocarriers integrated with dendrimers encapsulating photosensitizers are expected to be a clinically useful PS formulation for PDT and gene therapy.

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NANOMEDICINE

Nanocarriers shape up for long life

Keeping drug-delivery vehicles in the bloodstream for a long time is a challenge. New results suggest that adopting the filamentous shape of viruses may lead to better nanocarriers.

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Drug delivery is one of the most promising biomedical applications of nanotechnology, and several nanocarrier formulations, such as Doxil and Abraxane, have already been approved for clinical use in cancer chemotherapy[1]. A good nanocarrier is one that circulates in the blood for long periods of time and delivers the drugs with minimal side effects[2].

A number of popular nanocarriers based on liposomes[3] and polymeric micelles[4,5] can protect and deliver poorly soluble drugs effectively. These nanocarriers are typically between 100 and 200 nm in diameter, are spherical in shape, and are modified with poly(ethylene glycol) (PEG) to improve circulation in the bloodstream. Although PEG modification is an effective strategy, it cannot prolong the circulation of nanocarriers beyond 48 hours[3–5], and it is believed that those larger than 200 nm are cleared much faster than this.

On page 249 of this issue, Dennis Discher and colleagues[6] at the University of Pennsylvania report that filamentous polymeric micelles known as filomicelles (which are between 22 and 60 nm in diameter, and 2–8 μm in length) remain in the blood ten times longer than their spherical counterparts. By demonstrating the importance of shape, filomicelles should lead to new approaches for designing nanocarriers.

Block copolymers, which are composed of two or more different polymers, can spontaneously assemble into spherical, cylindrical, lamellar or vesicular shapes in selective solvents (Fig. 1)[7]. The chemical design of block copolymers brings several advantages because the size, stability, drug loading and release efficiencies can be controlled by simply varying the ratio of the polymer composition. Furthermore, it is also possible to build in smart functions such as tissue targetability and stimuli-responsiveness[5].

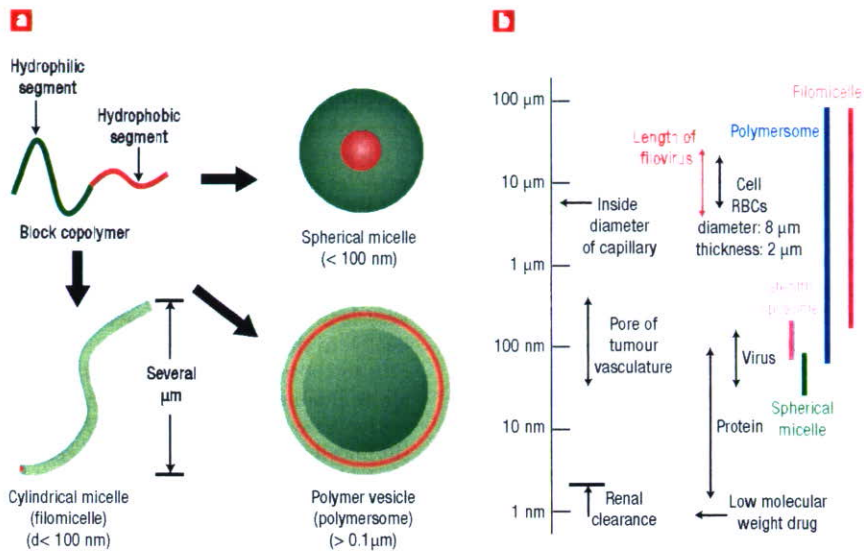

Figure 1 Block copolymers bearing a hydrophilic (green) and hydrophobic (red) segment can form different shapes and sizes. **a**, Depending on rigidity, length and ratio of the polymer segments, these synthetic copolymers can self-assemble into spherical micelles, polymer vesicles (polymersomes) or cylindrical micelles (filomicelles). Discher *et al.* showed filomicelles circulate longer than their spherical counterparts. **b**, Length scales showing how the various copolymer assemblies compare with structures in the body.

Since Kazunori Kataoka at the University of Tokyo[5] and, independently, Alexander Kabanov at University of Nebraska[8] reported the use of spherical micelles as drug vehicles in the late 1980s, block copolymer assemblies have gained increasing popularity in the field of drug delivery. To date, several micellar formulations of anticancer agents are in clinical trials[4,5]. In 2002, Discher and co-workers pioneered a new field of block copolymer assemblies[7]. They created polymer vehicles (polymersomes) and cylindrical micelles (filomicelles), and suggested that they could be used as nanocarriers and chemical reactors, and also as models to study biological systems[7]. Now they report the long circulating or 'super stealth' nature of filomicelles for the first time.

Inspired by the shape and the way filamentous viruses infect animals, Discher and co-workers decided to study how similarly shaped synthetic polymers circulate in the blood and, in particular, to explore how these structures fragment, flow and interact with cells. They found that the filomicelles persisted in the circulation of mice for up to one week, whereas PEG vesicles injected as controls cleared in two days. Interestingly, longer structures (~18 μm) were very quickly fragmented by flow effects and the activity of cells. This led to an optimal length that is approximately the same as the diameter of a biconcave red blood cell (8 μm), which are known to circulate for weeks. Shorter ones (~2 μm), on the other hand, fragmented more slowly and were gradually cleared from the blood.

These observations seem to indicate that shape and size may be important for prolonged circulation.

The soft structure of filomicelles might also influence blood circulation times. Rigid carbon nanotubes used for drug delivery are cleared from the circulation in hours[9]. Likewise, Discher and co-workers found that solid cylindrical micelles with a crosslinked core disappear in a similarly short amount of time.

When incubated with phagocytes — cells in the liver and spleen that remove foreign material — under flow conditions, long filomicelles stretched out along the streamlines of blood flow and were captured by cells less than their spherical counterparts. The shear forces on long structures overwhelmed the cells that are responsible for clearance, whereas smaller particles adhered to and were more easily taken up by the cells. Circulation times seem set by the ability of the structures to relax and/or fragment owing to flow or interactions with cells.

Surprisingly, other cells, such as the epithelial cells that line blood vessels and small cavities, also contribute to rapid shortening of the filomicelles. These cells imbibe the fragments in fluid-filled vesicles through a process known as pinocytosis. Such cellular uptake of material is intriguing but somewhat complicated. Moreover, integration of targeting ligands into filomicelles may further complicate the way they interact with cells both *in vitro* and *in vivo*.

Persistent circulation has many practical applications because these vehicles can increase exposure of drugs to cancer cells. Discher and co-workers loaded the filomicelles with paclitaxel — a common hydrophobic anticancer drug — and injected it into mice. They found that an eightfold increase in filament length at a given drug dosage showed the same relative therapeutic effect as an eightfold increase in paclitaxel dosage. Although this is a clear advantage, one is left to speculate if they are better than the liposomes and micelles that are currently used as stealth vehicles in clinical applications.

In particular, we wonder if these filomicelles can exit the blood vessels to reach tumour cells. It is known that blood vessels in tumours are characteristically 'leaky', in that they have pores (10s to 100s of nanometres) that allow nanoparticles to escape and accumulate in solid tumours[10]. The filomicelles that can circulate longer in the blood seem too large to pass through these pores, which may be a trade off to an effective accumulation in the tumours. Nonetheless, smaller micelles shed from the larger fragments may possibly accumulate in solid tumours. To answer these questions, a careful pharmacokinetic study — the distribution, metabolism and elimination — that directly compares filomicelles with other stealth vehicles is required.

Sustained drug release during blood circulation is beneficial from a clinical standpoint because patients can receive a single bolus injection instead of relying on long-term infusion. This approach can significantly improve the quality of life of patients receiving cancer chemotherapy. This work indicates that synthetic nanodevices inspired from natural systems can have surprisingly interesting properties and behaviours that could be about to change the way we design drug-delivery vehicles.

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ORGANIC ELECTRONICS

Memoirs of a spin

Relatively little is known about how spins interact with an organic environment. Now, a study of organic nanowires shows that spin information is preserved over exceptionally long times.

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Spins in solids tend to be forgetful. Even though the spin of an electron may be aligned along a given direction when the electron first enters a material, it will not take long before its orientation is lost. This tendency of spins to 'forget' their orientation varies from material to material. For example, metals are extremely good at washing out the alignment of a spin[1], whereas inorganic semiconductors are better at maintaining it[2]. But what about organic materials? On page 216 of this issue, Supriyo Bandyopadhyay and co-workers[3] from the Virginia Commonwealth University and the University of Cincinnati in the US report that spins in organics can remember their direction over exceptionally long times.

The electron spin is the ultimate information bit because its orientation is quantized and can only point up or down. Spin logic will require extremely low power compared with transistors because the energy needed to flip a spin is orders of magnitude smaller than that needed to drive an electric current. Finally, magnetic materials provide an endless source of aligned spins and are already in use in a number of magnetoelectronic devices. If all of these tantalizing properties are realized in organic materials, one could foresee a new generation of plastic devices based entirely on electron spins as the active memory elements. Moreover, from the perspective of fundamental research, we can expect the discovery of new phenomena solely related to spin transport in organic media.

日本骨粗鬆症学会 骨Paget病の診断と治療ガイドライン委員会報告

わが国における骨パジェット病の有病率と臨床的特徴

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はじめに

骨パジェット病(Paget's disease of bone)は局所での骨リモデリングの異常により，骨微細構造の変化と骨の形態的な腫大・変形とそれに伴う局所骨強度の低下をきたす疾患である。1877年，英国のJames Paget卿により変形性骨炎(osteitis deformans)として初めて詳細が報告された[1)]。その中で病理組織学的特徴からchronic inflammationと表現したところは，非炎症性に骨リモデリングの異常を局所的にきたす疾患であるという現在の認識と異なる点があるが，「変形性」という表現は今なおX線像上の読影を行ううえで有用であり，その病像をとらえた的確な表現である。有病率の高い英国では加齢とともに，使用していたシルクハットが入らなくなり，頭蓋骨の変形に気づかれることがしばしば紹介される。病名に関してわが国ではさまざまないきさつで誤った発音で記載された経緯があるが，Pagetの発音はパジェットが正しく，骨パジェット病という正しい病名で述べられることが望まれる[2)]。

骨パジェット病は英国を含め欧州の南地域や米国には極めてありふれた高齢者に多い骨疾患であり，英国ではThe National Association for the relief of Paget's disease(NARPD)という協会が1973年に，米国ではThe Paget foundation for Paget's disease of bone and related disordersという財団が1978年に設立され，現在ではインターネットを通じて患者および医療関係者が容易に骨パジェット病に関する情報を手にすることができるようになっている。一方，わが国では極めて珍しい疾患であり一部の医師以外は患者を診る機会がほとんどないが，わが国でも多数の貴重な症例報告が蓄積され，骨パジェット病に罹患しさまざまな合併症をきたしている患者の存在は知られていた。しかし，わが国での罹患率や臨床的特徴に関しての情報や，一般の医師の診断機会を増やすための啓蒙資料もない状況が長く続いていた。このような背景のなか2002年に日本骨粗鬆症学会の中で，当時の日本

Prevalence and Clinical Features of Paget's Disease of Bone in Japan

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日本骨粗鬆症学会骨Paget病の診断と治療ガイドライン委員会 [1)] 大阪大学大学院医学研究科器官制御外科学 [2)] 徳島大学大学院運動機能外科 [3)] 大阪市立大学大学院医学研究科代謝内分泌病態内科学 [4)] 東京大学医学部22世紀医療センター・関節疾患総合研究講座 [5)] 兵庫医科大学篠山病院整形外科 [6)] 慶応義塾大学医学部整形外科 [7)] 帝京大学医学部整形外科 [8)] 和歌山県立医科大学放射線部 [9)] 大日本住友製薬株式会社 [10)] 川崎医科大学医学部放射科核医学 [11)] 日本骨粗鬆症学会

表1　骨Paget病の診断と治療ガイドライン委員会

回	時　期	場　所
1	平成14年8月18日(日)	ホテルラフォーレ新大阪
2	平成14年11月22日(木)	ホテル日航東京
3	平成15年6月2日(月)	大阪国際会議場
4	平成15年12月8日(月)	ホテルラフォーレ新大阪
5	平成16年3月8日(土)	銀行倶楽部
6	平成16年8月4日(水)	リーガロイヤルホテル
7	平成16年11月18日(木)	大宮ソニックシティビル

骨粗鬆症学会理事長 故森井浩世名誉教授の発案により,「骨Paget病の診断と治療ガイドライン委員会(委員長 吉川秀樹大阪大学整形外科教授)」が設けられた。その第1回委員会で活動の目的を,下記の4点とすることが確認された。

①骨パジェット病の診断・治療上の留意点を一般臨床医に示す。

②骨パジェット病のわが国と欧米との差を発症率以外の点につき明らかにする。

③症状やADL障害の程度,病的骨折,腫瘍など二次的な障害の頻度を調べ,治療のエンドポイントと指針を示す。

④新しい治療法のわが国への導入の必要性を検討する。

計7回の委員会(**表1**)が開かれ,骨パジェット病に関する全国レベルの有病率と臨床的特徴に関する調査,またわが国での診断と治療のガイドライン作成が行われ,二つのレポートとしてまとめられた[3,4]。またその診断の手助けのためのX線像アトラスの作成が行われ,「骨パジェット病アトラス」(日本骨粗鬆症学会:骨Paget病の診断と治療ガイドライン委員会編・著)[5]として出された。さらに委員会活動を通して認識されるに至った欧米での第一選択薬である高用量ビスフォスフォネート剤の必要性を本誌に公表し[6],共鳴いただいた企業の協力のもと希少疾病用医薬品の指定を受け,リセドロネート高用量の治験が開始されるに至った。ここでは,骨パジェット病に関する全国レベルの有病率と臨床的特徴に関する調査の結果を報告する。

わが国での有病率の検討

まず,本委員会では1990～2002年の間に報告されたわが国の骨パジェット病の症例を医学中央雑誌で検索を行い,その内容をすべて確認し,罹患部位,年齢,性別,施設などの情報から重複症例を除外して得られた154例の症例を報告科別に分類した。この結果,72.1%が整形外科からの報告であったので,整形外科を対象とした全国調査を行うことに決定した。日本整形外科学会の了承を得たうえで,日本整形外科学会認定施設2,320病院の整形外科宛に,現在通院中の骨パジェット病症例の有無とその症例数を確認する調査を郵送法で行った。75.4%の回答を得て,インド人症例1例を含む194名の患者が通院中であることが確認できた。以上の,過去の症例報告の72.1%が整形外科からであった点,一次調査の返答率75.4%,日本人患者193名が現在整形外科を通院中,2002年の日本の人口126,008,000人から計算すると,100万人に2.8人の有病率であることが明らかとなった。アジア,アフリカ地域では,骨パジェット病の有病率が極めて低いといわれてきたが,具体的な数値としてわが国での有病率の低さが初めて示された。北欧をのぞいたヨーロッパ諸国,米国,オーストラリア,ニュージーランドでは,骨パジェット病の有病率が高く,0.1～5%の有病率と報告されている。これに比較してわが国では格段に低い有病率である。

わが国の骨パジェット病患者の臨床的特徴

二次調査を,診断方法,年齢,性別,家族歴,

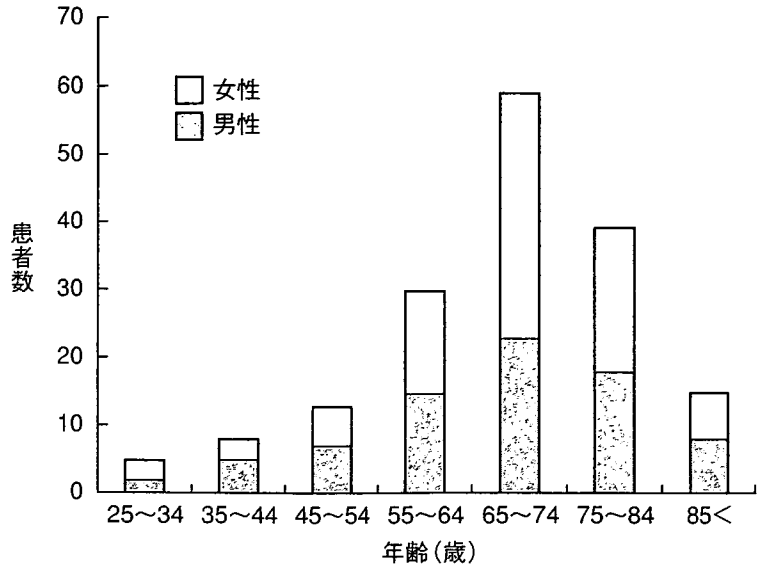

図1　わが国での骨パジェット病患者の年齢分布(文献3から引用)

自覚症状，骨折などの合併症，罹患部位，血清アルカリフォスファターゼ(ALP)レベル，治療薬，予後について行い，170名(インド人症例1例を含む)の情報を得ることができ，日本人患者169名のデータを解析した。その結果明らかとなった，患者の年齢分布を**図1**に示す。わが国の患者の平均年齢は64.7歳で，患者の90%以上が45歳以上であった。高齢者に多いこの年齢分布様式は骨パジェット病の有病率の高い国と大差がない。

わが国での男女比は0.86：1でやや女性に多く(**表2**)，1.2～1.8の比で男性に多いという頻度の多い欧米諸国での報告と異なる傾向であった。患者の家族集積性に関してはわが国では6.3%で，ほとんど骨パジェット病の患者が散発性発症であり，欧米での家族集積性が15～40%の頻度であることに比較して少ない。欧米では無症候性のものが多く，有症状の患者は多い報告でも約30%程度であるが，わが国での調査では75.1%が有症候性であった。この大きな差の説明として，有病率の少ないわが国では診断に慣れておらず無症候性の患者が診断されずに見過ごされている可能性と，わが国では希少疾患ゆえ治療薬の認可が遅れており，十分なコントロールができない例があることなどが考えられる。症状は腰痛，股部痛，骨格変形，臀部痛，膝痛，難聴，かみ合わせの異常や開口障害などの歯科的な障害の順に多く(**表3**)，欧米と差はない。骨パジェット病には，罹患骨が1ヵ所に

表2　わが国の169名の骨パジェット病患者の臨床像(文献3から引用)

項　目	
年齢(年)(Mean±SD)	64.7 ± 14.5
男性/女性(比)	78:91 (0.86:1)
家族集積性(n＝158)[a]	10 (6.3%)
自覚症状	
無症候性(%)	42 (24.9%)
有症候性(%)	127 (75.1%)
単骨性/多骨性(比)	87:82 (1.06:1)
罹患部位	
骨盤	93 (55.0%)
脊椎	54 (32.0%)
大腿骨	46 (27.2%)
頭蓋骨	34 (20.1%)
脛骨	25 (14.8%)
血清ALP値(n＝164)[b]	
正常範囲	17 (10.4%)
正常上限値の3倍以下	74 (45.1%)
3～6倍	49 (29.9%)
6倍以上	24 (14.6%)

ULN：正常上限値，[a]：患者11名のデータなし，[b]：患者5名のデータなし

表3　有症候性骨パジェット病患者の127名にみられた症状(文献3から引用)

症　状	症例数
腰痛	30
股関節痛	29
骨格変形	24
臀部痛	18
膝痛	11
難聴	8
歯科的な障害	5
その他	9

限局されるは単骨性と，離れたいくつかの骨が罹患する多骨性がある。わが国では単骨性と多骨性がほぼ同等の頻度で，欧米諸国で多骨性の頻度が66％とする報告に比較してやや低かった。罹患部位は骨盤が約半分の患者で罹患する最も頻度の高い部位であり，次に脊椎，大腿骨，頭蓋，脛骨の順であった。この点は頻度の多い欧米諸国と同様である。血清ALP値は約9割の患者で正常上限を超える高値であった。

骨悪性腫瘍の発生は3例(1.8％)の頻度で欧米諸国と同等であった。一方，大腿骨罹患患者の約2割強の患者に骨折を生じており，欧米での大腿骨罹患患者での骨折率に比較して高かった。

診断法に関しては，有病率の高い欧米諸国では診断をX線像と血清ALP値で行うことが勧められており，出血などの合併症もある骨生検は通常行われない。わが国では55％の患者で骨生検が行われていた(**表4**)が，希少疾患のためにX線像上の診断に不慣れであるための結果と考えられる。わが国では骨パジェット病の治療薬として認可されている薬物療法はエチドロネートとカルシトニンだけであるが，12％以上の患者に他の未認可のビスフォスフォネートが用いられていた。エチドロネートとカルシトニンでは十分な治療効果を得ることができなかった症例が存在することが推察された。

表4　わが国での骨パジェット病患者の治療，予後と合併症(文献3から引用)

項　目	頻度(％)
診断法	
骨生検	93 (55.0)
骨生検なし	76 (45.0)
薬物治療	
非投与	26 (15.4)
NSAIDs	65 (38.5)
カルシトニン	105 (62.1)
エチドロネート	25 (14.8)
アレンドロネート	15 (8.9)
リセドロネート	2 (1.2)
他のビスフォスフォネート剤	4 (2.4)
予後($n=152$)[a]	
無症候	57 (37.5)
疼痛減少	50 (32.9)
疼痛持続	25 (16.4)
合併症	20 (13.2)
合併症(内訳)	
骨折	16 (9.5)
大腿骨	10
骨盤	2
腰椎	1
脛骨	1
報告なし	2
人工股関節全置換術	1 (0.6)
肉腫	3 (1.8)

[a]：患者17名のデータなし
薬物療法は使用歴を含めた延べ使用頻度を示している。

おわりに

本委員会での有病率調査により骨パジェット病はわが国では大変頻度が少ないことが確認されたが，十分な治療がなされることなく，変形が進行し骨折を繰り返し，苦しんでいる患者がいることも明らかとなった。珍しい疾患であるがゆえに診断されることのないままになっている患者もいると考えられる。罹患患者がいる以上は，頻度は少なくとも的確な診断と治療をできる状況をわが国でも確立することが重要である。委員会活動により骨パジェット病の診断と治療のガイドライン，骨パジェット病アトラスが出され，また高用量リセドロネートの治験

も始まり，今まさにそのような状況が確立されつつある。委員会発足を企画され委員会にも参加されていた故森井浩世理事長の遺志を継ぎ，骨パジェット病患者に福音がもたらされるべく今後これらの成果を最大限に活用する努力が必要である。

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日本骨粗鬆症学会 骨Paget病の診断と治療ガイドライン委員会報告

骨Paget病の診断と治療ガイドライン委員会成果報告

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要 約

日本骨粗鬆症学会骨Paget病の診断と治療ガイドライン委員会は，ここに骨パジェット病の診断と治療のガイドラインを提示する。本ガイドラインは，骨パジェット病の疫学，病理病態，臨床症状，診断そして整形外科的治療を含めた治療に関わる情報を提供するものである。

骨パジェット病は，代謝性骨疾患である。その特徴的な病態は，骨吸収の異常な亢進と，それに続く旺盛な骨形成である。

主訴は，骨病変由来の疼痛であることが多い。稀に，罹患骨から悪性骨軟部腫瘍の発生をみることがあり注意を要する。

骨パジェット病は，欧米に比べ，わが国では稀な疾患である。欧米では，代謝性骨疾患のなかでは，骨パジェット病は骨粗鬆症についで患者数が多い疾患であるため，単純X線写真での典型的な異常所見と，血清アルカリフォスファターゼ(ALP)値の高値をもって診断を確定できることが多い。一方，罹患頻度の低いわが国では，骨生検を実施して組織学的診断がなされることが多い。骨シンチグラムにおける骨病変部に一致した異常集積像，血清ALP値の高値は，骨パジェット病の診断を確実にする。

骨パジェット病の治療適応は，骨病変由来の疼痛がある場合である。わが国で認可されている骨パジェット病の治療薬は，エチドロネートとカルシトニンにすぎず，欧米に比べて治療薬の選択肢が少ない。現在，わが国では，骨パジェット病の新たな治療薬としてリセドロネートの開発が進められている。骨パジェット病の外科的治療は，不安定性骨折，変形性関節症，悪性骨軟部腫瘍そして骨変形がみられる場合にのみ適応となる。

はじめに

骨パジェット病は，1877年，英国の外科医であり病理学者であったSir James Pagetが，変形

Guidelines for Diagnosis and Management of Paget's Disease of Bone in Japan

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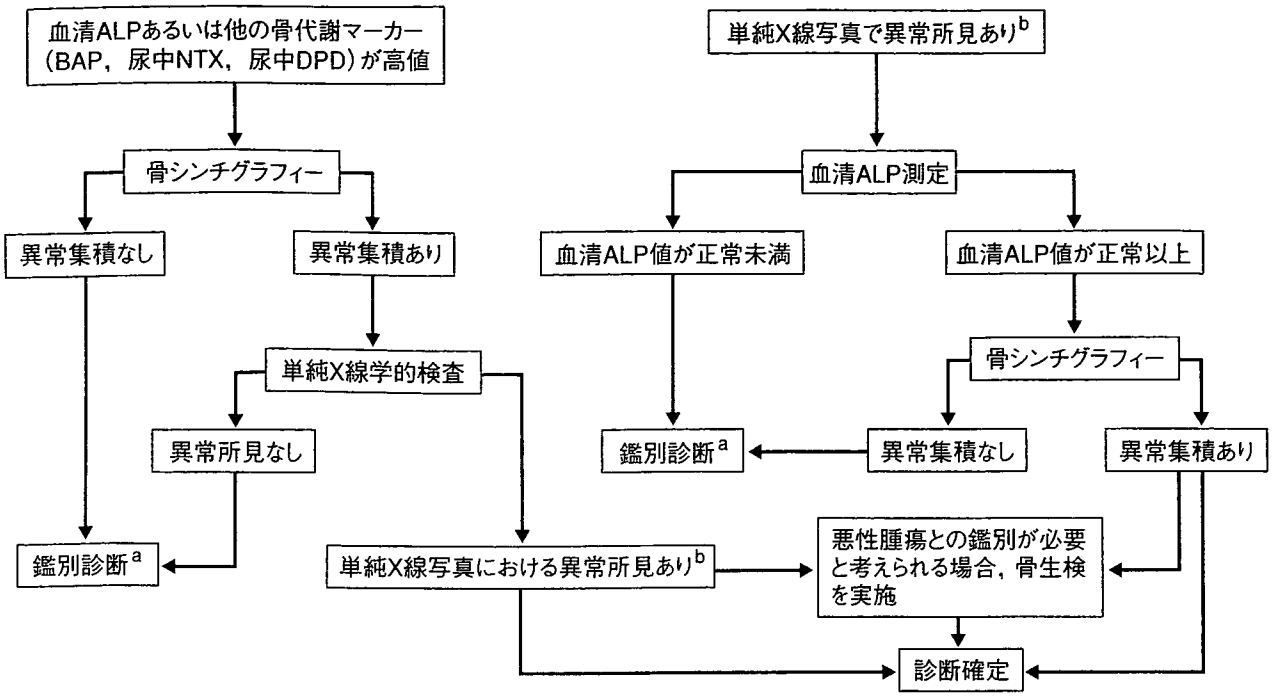
日本骨粗鬆症学会骨Paget病の診断と治療ガイドライン委員会 [1)]徳島大学医学部運動機能外科 [2)]大阪大学大学院医学研究科器官制御外科学 [3)]大阪市立大学大学院医学研究科代謝内分泌病態内科学 [4)]東京大学医学部22世紀医療センター・関節疾患総合研究講座 [5)]兵庫医科大学篠山病院整形外科 [6)]慶応義塾大学医学部整形外科 [7)]帝京大学医学部整形外科 [8)]川崎医科大学医学部放射科核医学 [9)]和歌山県立医科大学放射線部 [10)]大日本住友製薬株式会社 [11)]日本骨粗鬆症学会



ALP：alkaline phosphatase，BAP：bone-specific alkaline phosphatase，
NTX：type I collagen cross-linked N-telopeptides，DPD：deoxypyridinoline

[a] 骨パジェット病の鑑別診断では，前立腺癌や乳癌などの骨転移や骨硬化を示す骨系統疾患が含まれる。
[b] 骨パジェット病のよく知られた単純X線写真での特徴は，骨吸収の亢進，骨梁の粗造化や際立ち，骨皮質の肥厚，骨の輪郭の拡大である

図1　骨パジェット病診断のフローチャート(文献10から引用改変)

性骨炎として初めて記述した[1]。骨パジェット病は，欧米では骨粗鬆症に次いで罹患頻度が高い代謝性骨疾患であるが，わが国では稀な疾患である[2〜5]。

骨パジェット病の特徴的病態は，異常に亢進した骨吸収と，それに続く旺盛な骨形成にある。骨パジェット病では，骨病変の拡がりに応じて症状が出現するが，無症状であることが多い[4]。その合併症のなかには，極めて稀に悪性骨軟部腫瘍が発生することがあるため，注意を要する。

疫　　学

わが国の疫学調査(一次調査)では骨パジェット病の患者数が193名であった[6]。すなわち，わが国における骨パジェット病の有病率は人口10万人あたり0.28人である。しかし，年齢が55歳以上となれば，その有病率が人口10万人あたり0.41人と上昇する。

骨パジェット病は，わが国では稀な疾患である。しかし，スカンジナビア半島の諸国を除いたヨーロッパ諸国，オーストラリアおよびニュージーランド，北米では，骨パジェット病は，ごくありふれた骨疾患である[2]。以上の疫学調査から，骨パジェット病の病因には，遺伝因子と環境因子の双方が関わっていることがわかった。

骨パジェット病は，加齢とともに罹患頻度が高まる。わが国の疫学調査(二次調査)では，骨パジェット病患者の平均年齢は64.7歳であった。その男女比は0.86：1であり，骨パジェット病は女性に多く発症する傾向がみられた[6]。

病態生理学

骨パジェット病では，患者の地理的あるい