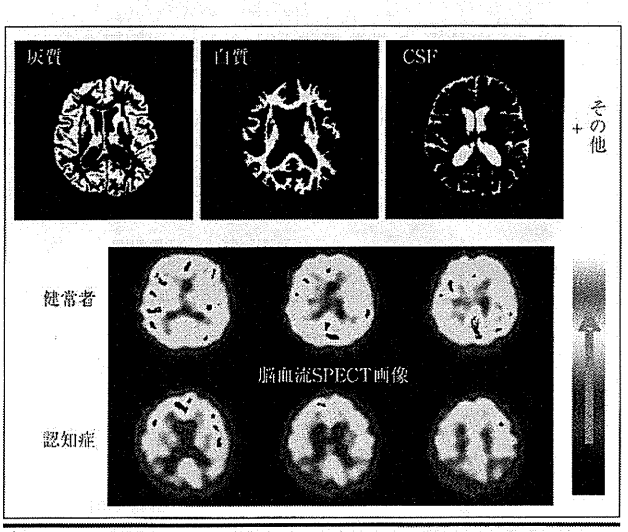

図2　人間の脳の構造(上)，健常者と認知症患者のSPECT画像の比較(下)

2 脳血流SPECT画像の解析手法

2.1 基礎知識[7]

まず，認知症の画像診断に関する基礎知識を説明する．図2(上)に示すように，人間の脳は，灰質・白質・CSF(脳脊髄液)・その他の四つの領域から構成されている．健常者の場合，灰質と白質の血流値はほぼ一定で，白質の血流値は灰質の約4倍である．また，CSFとその他の領域の血流値はほぼゼロである．そして，アルツハイマー病・レビー小体型認知症・脳血管性認知症などの典型的な所見として，図2(下)に示すように灰質に血流が低下した部位が現れることが挙げられる．したがって，認知症の画像診断では，I-123などの血液に集積する放射性薬剤を用いて，脳血流SPECT画像を撮影して，医師の読影や画像解析ソフトウェアにより，血流低下部位を発見することにより診断が行われる．以降で説明する画像解析手法は，いずれも灰質における血流低下部位の発見を目的としたものである．血流低下が生じる部位は認知症の種類と相関があり，例えば，アルツハイマー病の場合は，頭頂葉や後部帯状回と呼ばれる部位に血流低下が生じやすいことが知られている．

2.2 統計学的画像解析法[1)~4)]

次に，認知症の画像診断への実用化が進んでいる機能画像解析ソフトウェアであるSPMと3D-SSPについて説明する．両者とも同じ原理に基づいているため，総称して統計学的画像解析法と呼ばれることも多く，ここでは3D-SSPを例に取って説明を行う．統計学的画像解析法では，事前に健常者SPECT画像のデータベースを構築しておき，患者と健常者のSPECT画像を統計的に比較することで，血流低下部位を検出する．ただし，脳形状には個人差が存在するので，その影響を軽減するため，すべてのSPECT画像をTalairachの標準脳座標系と呼ばれる標準的な脳形状を表す座標系に非線形変換した上で，比較が行われる．この脳形状を統一化する処理を解剖学的標準化と呼ぶ．すなわち，「脳形状を統一化して患者と健常者のSPECT画像を比べれば，血流低下が検出できる」というのが基本となる考え方である．具体的な3D-SSPの処理手順をまとめると，以下のようになる．

STEP 1：事前に，年齢・性別・SPECT装置・撮影条件ごとに，健常者SPECT画像のデータベースを構築しておく．

STEP 2：全画像をTalairachの標準脳座標系に非線形変換して，血流値を基準部位(小脳など)の濃度値が同じになるように正規化する．

STEP 3：患者と健常者のSPECT画像を統計的に比較して，次式で定義されるZスコアと呼ばれる量を算出する．

$$Z(x,y,z)=\left[m(x,y,z)-f(x,y,z)\right]/\sigma(x,y,z) \quad (1)$$

ただし，

$f(x, y, z)$＝患者のSPECT画像

$m(x, y, z)$＝健常者のSPECT画像の平均

$\sigma(x, y, z)$＝健常者のSPECT画像の標準偏差

である．その定義から，Zスコアは，患者の血流量が健常者の血流量の標準偏差の何倍低下しているかを表す数値である．

STEP 4：Zスコアを医師にわかりやすく表示する．具体的には，単純に各横断面を表示する手法の他に，脳表から一定深さ範囲のZスコアの最大値を求め上下前後左右から最大輝度投影(Maximum Intensity Projection)する手法が用いられる．

図3に，典型的なアルツハイマー病患者のSPECT画像を3D-SSPで処理した結果の例を示す．認知症患者の場合は，概ね健常者と比較して血流低下量が大きく算出される．統計学的画像解析法は，機能画像を用いた診断へ画像解析を

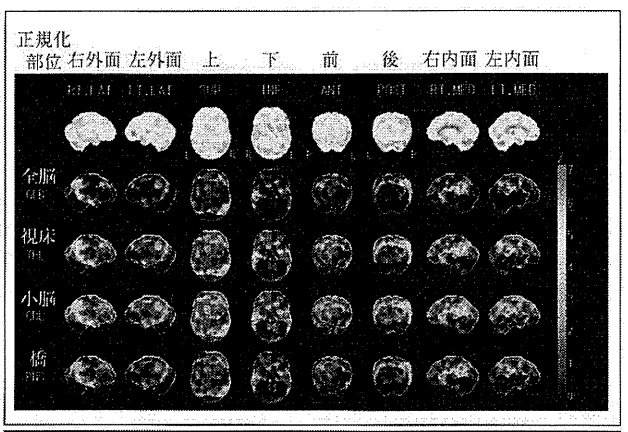

図3　3D-SSPによるアルツハイマー病患者のSPECT画像の処理結果

用いる道を切り開いた意味で画期的なものであった．実際に，2000年以降臨床現場での実用化が進み，統計学的画像解析法での利用を目的とした，大規模健常者データベースの構築プロジェクトも開始されている．一方，統計学的画像解析法の問題点としては，①健常者データベースの構築に手間がかかること，②解剖学的標準化により脳形状個人差の影響を完全になくすことはできず誤差が生じること，が挙げられる．実際に，脳萎縮が進行してTalairachの標準脳と大きく異なる脳形状の症例の場合には，解剖学的標準化の誤差が大きくなる問題点が知られている．

2.3　MRI情報を用いた解析手法FUSE[5)6)]

筆者らは，2004～2006年に，上述の統計学的画像解析法の問題点を克服する，新しい脳血流SPECT画像解析手法の開発を行った．目標とした点は，健常者データベースを使用せずに，かつ，脳形状個人差の影響をまったく受けずに，血流低下量分布を算出することである．以降では，その新しい解析手法FUSEについて説明する．

まず，FUSEでは，健常者SPECT画像のデータベースを用いずに，同一患者のSPECT画像とMRI画像の2枚のみを用いて解析を行う．すなわち，「同一患者のSPECT画像とMRI画像を比較して血流低下を検出する」原理に基づいている．もちろん，同一患者であっても，SPECT画像とMRI画像の領域ごとの濃度値はまったく異なるため，単純に両者を比較することはできない．そこで，最初にMRI画像を解析して，患者の血流が正常と仮定した時のSPECT画像を模擬したテンプレート画像を作成して，これと実測のSPECT画像を比較して，血流低下部位を検出する．FUSEの具体的な処理手順をまとめると，以下のようになる．

STEP 1：MRI画像に領域分割などの画像処理を施して，患者の血流が正常と仮定した時のSPECT画像を模擬したテンプレート画像$f_{temp}(x, y, z)$を作成する（具体的な処理手順は後述する）．

STEP 2：実測のSPECT画像$f_{sp}(x, y, z)$とテンプレート画像$f_{temp}(x, y, z)$の位置合わせを行う．位置合わせは，両者の相互相関係数を最大化する剛体変換で充分である．

STEP 3：血流低下量の分布を表すDSI画像（Deterioration Score Image）を，次式で算出して，表示する．

$$DSI(x,y,z)=f_{temp}(x,y,z)-f_{sp}(x,y,z) \tag{2}$$

次に，FUSEにおいて最も重要なテンプレート画像作成の手順を述べる．テンプレート画像の作成は，健常者の場合は灰質・白質の各領域の血流値は一定，CSFとその他の領域の血流値はゼロの仮定の下で，以下の手順により行う．

STEP 1：MRI画像から骨と皮膚の領域を除去する．

STEP 2：MRI画像を灰質・白質・CSF（脳脊髄液）・その他の四つの領域に領域分割する．

STEP 3：血流量の基準部位とするため，小脳の領域を同定する．

STEP 4：次式によりテンプレート画像$f_{temp}(x, y, z)$を作成する．

$$f_{temp}(x,y,z)=h_\sigma(x,y,z)*\left[m_W I_W(x,y,z)+m_G I_G(x,y,z)+m_S I_S(x,y,z)\right] \tag{3}$$

m_W =白質の血流値
m_G =灰質の血流値
m_S =小脳の平均血流値
$I_W(x,y,z)$ =白質のIndicator Function
$I_G(x,y,z)$ =灰質のIndicator Function
$I_S(x,y,z)$ =小脳のIndicator Function
$h_\sigma(x,y,z)$ =半値幅2.35482のガウスフィルタ

ただし，*は畳み込み積分を表す．Indicator Function $I_W(x, y, z)$, $I_G(x, y, z)$, $I_S(x, y, z)$は，各々白質・灰質・小脳の領域内の画素に値1をそれ以外の領域の画素に値0を代入した2値画像で，"STEP 2"における領域分割結果と"STEP 3"から得られる．また，式(3)においてガウスフィルタで平滑化を行う理由は，テンプレート画像の解像度を実測のSPECT画像に一致させるためである．なお，血流値m_W, m_G, m_Sの値は，多数の症例を処理した結果同じ装置でも個人差が無視できないことがわかり，症例ごとに適切な値を推定するヒストグラムマッチング法と呼ばれる手法を開発した[5)6)]．

STEP 5：大脳全体の血流値が小脳と比較して低下している場合を考慮して，小脳の血流値の平均と大脳の血流値の平均が一致するように大脳全体の血流値を正規化する．

図4に，アルツハイマー病患者のSPECT画像をFUSEで処理した結果の例を示す．概ね医師の目視と合致した解析結果が得られている．また，25症例の臨床データを用いた3D-SSPとFUSEの比較実験の結果が，文献5) 6)に示されている．その結果によれば，3D-SSPとFUSEの処理結果の間には非常に強い相関があり，FUSEの精度や信頼性は3D-SSPとほぼ同等と評価されている．

2.4　SPECT画像の画質改善[6)8)]

画像生成の過程において，SPECT画像は，部分容積効果・散乱線・γ線吸収などのさまざまな要因による画質劣化を受けている．近年，これらの画質劣化を補正する研究が精力的に行われているが，未だに定量性や分解能が高い高画質のSPECT画像を得るのは難しいのが現状である．特に，認知症やてんかんの診断においては，部分容積効果に

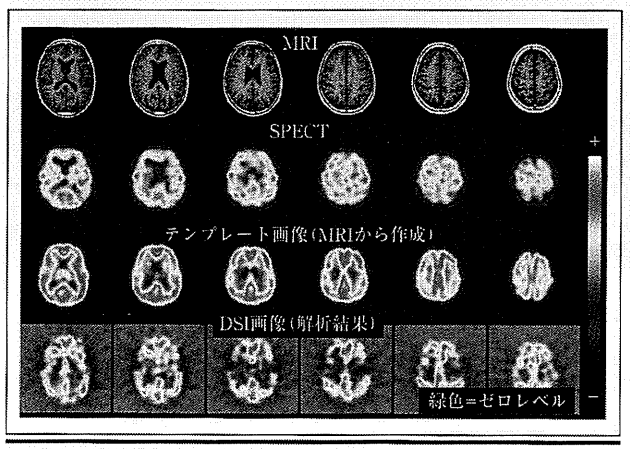

図4　FUSEによるアルツハイマー病患者のSPECT画像の処理結果

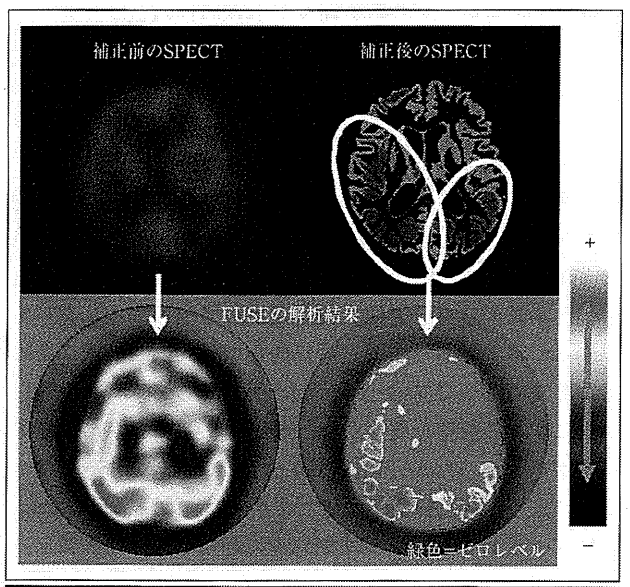

図5　部分容積効果補正前と補正後のSPECT画像とFUSEによる解析結果

よる画像のぼけが大きな問題になる．部分容積効果とは，検出器のぼけ特性によりSPECT画像がぼけて定量性が低下する現象のことである．単に画像がぼけて血流低下部位が見にくくなるだけではなく，その影響により健常者であっても灰質の血流値に均一性がなくなり，部分容積効果による偽の血流低下か真の血流低下かの判断がつかなくなる．部分容積効果を補正する手法については，文献8）に詳しい解説がある．具体的には，①デコンボリューションによるぼけ補正，②同一患者のMRI画像を用いるMuller-Gaertnerの手法，などの補正法が存在する[8]．図5に，筆者らが開発した新手法を用いて部分容積効果の補正を行った結果を示す[6]．ただし，補正前と補正後のSPECT画像をFUSEで解析した処理結果も合わせて示してある．なお，この手法は，同一患者のMRI画像を用いる補正法である．画質改善の効果は驚くほど大きいことがわかる．

3 むすび

本稿では，機能画像を対象とした計算機支援診断の中で早くから研究が行われ，いち早く実用化された脳血流SPECT画像の解析を取り上げた．具体的には，すでに実用化が進んでいる統計学的画像解析法と，筆者らが開発したFUSEについて解説した．脳機能のイメージングや画像解析を取り扱う分野は，NeuroimagingやBrain Mappingなどと呼ばれ，医学系では重要視されている大きい分野であるが，工学系からの寄与が小さいようである．本稿を契機として，より多くの技術者に興味を持っていただけたら幸いである．

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〔文　献〕

1) K.J. Friston, A.P. Holmes, K.J. Worsley, J.-P. Poline, C.D. Frith and R.S.J.Frackowiak: "Statistical parametric maps in functional imaging: A general linear approach", Human Brain Mapping, 2, 4, pp.189-210 (Oct. 1994)
2) K.J. Friston, J.T. Ashburner, S.J. Kiebel, T.E. Nichols and W.D. Penny: "Statistical parametric mapping: The analysis of functional brain images", Academic Press (2006)
3) S. Minoshima, K.A. Frey, R.A. Koeppe, N.L. Foster and D.E. Kuhl: "A diagnostic approach in Alzheimer's disease using three-dimensional stereotactic surface projection of fluorine-18-FDG PET", J. Nucl.Med., 36, 7, pp.1238-1248 (July 1995)
4) 内田佳孝，伊東久夫，飯森隆志，木川隆治，岡田真一，簑島聡："統計学的画像診断（3D-SSP）"，日本放射線技術学会雑誌，58，12，pp.1563-1572 (Dec. 2002)
5) H. Kudo, T. Asada and T. Takeda: "Analyzing cerebral blood-flow SPECT images for the diagnosis of dementia: A new approach FUSE", 日本医用画像工学会誌, Medical Imaging Technology, 26, 3, pp.169-174 (May 2008)
6) 工藤博幸：　"MRI情報を用いた脳血流SPECT画像の解析と再構成"，日本医用画像工学会誌, Medical Imaging Technology, 28, 1, pp.19-25 (Jan. 2010)
7) 松田博史，朝田隆：　"見て診て学ぶ痴呆の画像診断"，永井書店 (2004)
8) H. Zaidi: "Quantitative analysis in nuclear medicine imaging", Springer Verlag (2005)

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Research Paper

Accelerated Blood Clearance Was Not Induced for a Gadolinium-Containing PEG-poly(L-lysine)-Based Polymeric Micelle in Mice

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Purpose. Accelerated blood clearance (ABC) is induced by repeated injections of PEGylated liposomes. In this study, the ABC was investigated for a gadolinium-containing PEG-poly(L-lysine)-based polymeric micelle (Gd-micelle) and PEGylated liposome (Gd-liposome) in mice.
Materials and Methods. Effects of the first injection of Gd-micelle on the tissue distribution of the second dose of Gd-micelle were studied. Additionally, effects of the first injection of Gd-micelle, Gd-liposome, empty liposome, polyethyleneglycol ($PEG_{500,000}$), and PEG-lipid on the distribution of the second dose of the Gd-liposome were evaluated.
Results. Results indicated that the tissue distribution of the second injection of the Gd-micelle at a dose of 33, 5, or 2 μmol Gd/kg was not affected by the first injection of the Gd-micelle at different doses and time intervals or of the empty PEGylated liposome 7 days before. ABC of Gd-liposome at a dose of 2.3 μmol Gd/kg (corresponding to 10 μmol lipids/kg) was observed when the empty PEGylated liposome or Gd-liposome, but not the Gd-micelle, $PEG_{500,000}$ or PEG-lipid, was pre-administered.
Conclusions. The hydrophobic core of the micelle or lipid bilayer of PEGylated liposome has a major effect on this phenomenon. These studies have significant implications for the evaluation of PEG-poly (L-lysine)-based micellar formulation of Gd-based contrast agents.

KEY WORDS: accelerated blood clearance; gadolinium; PEGylated liposome; polyethylene glycol (PEG); polymeric micelle.

INTRODUCTION

Long-circulating liposomes with surface-modified polyethyleneglycol (PEG) are often used as carriers of therapeutic agents, since they avoid capture by the reticuloendothelial system (RES) and can extend the systemic circulation time of agents, thereby improving drug delivery (1,2). It was hypothesized that PEG on the surface of liposomes forms a water shell, resulting in decreased adsorption of opsonins and subsequent phagocytosis by cells of the RES (3,4). However, PEGylated liposomes are known to lose their long-circulating property with multiple dosing. Recently, it has been reported that the first dose of PEGylated liposomes injected intravenously caused a loss of the long-circulating property and extensive accumulation in the liver at the second dose injected several days later in mice, rats, rabbit, and rhesus monkeys (5–11), a phenomenon known as accelerated blood clearance (ABC). Besides PEGylated liposomes, other nanocarriers, such as nanoparticles containing PEG, also produced this phenomenon (12). Therefore, ABC would have a significant impact on the application of long-circulating liposomes and nanoparticles with multiple administrations. In clinical applications of liposomal carriers, Gabizon *et al.* reported a reduced clearance of doxorubicin-containing PEGylated liposome in the repeated injections. This opposite behavior to the ABC phenomenon resulted from toxic activity of the encapsulated doxorubicin against the RES (13). Presently, the ABC phenomenon is not a problem in a cancer chemotherapy by the use of a PEG-liposomal carrier, whereas the ABC phenomenon in human clinics must be important for less toxic drug or gene delivery applications of the PEGylated liposomes.

To date, studies of ABC have focused mainly on PEGylated liposomes. Many factors can affect the extent to which ABC is induced by PEGylated liposomes. First of all, the dose of lipid plays an important role, with ABC enhanced at lower concentrations of lipid (6,7,12). Second, ABC occurs in a time-dependent manner (5,7). The time interval between the first and second doses is a key factor. Third, when the amount of PEGylated lipid in the first injection was ≤5 mol%, the second dose of PEGylated liposomes was eliminated more quickly from plasma than liposomes containing >10 mol% PEGylated lipid injected as a first dose (7,8). In addition, the ABC phenomenon

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was reported to be independent of liposomal size, surface charge, and PEG molecular weight (5,7,8).

During the past decade, polymeric micelles, supramolecular assemblies of block copolymers, have demonstrated their utility in drug delivery systems and are currently recognized as promising nanocarriers for enhancing the efficacy of drugs and genes (14–16). Since ABC has a considerable impact on the multiple drug administration, it is necessary to study whether the phenomenon is induced by repeated injections of polymeric micelles. Gadolinium (Gd)-based contrast agents are widely used in magnetic resonance imaging (MRI) to improve the conspicuity of lesions or visualization of blood vessels (17). However, these agents are rapidly cleared from the circulation. To overcome this problem, nanocarriers, such as liposomes and polymeric micelles, are used to encapsulate the agents so as to prolong their circulation and allow them to accumulate in tumors for diagnosis (18–20). If polymeric micelles containing a diagnostic agent cause the ABC phenomenon, then circulation time will be reduced after a second dose and the accuracy of the diagnosis will be affected. Furthermore, polymeric micelles containing MRI agents or drugs administered during diagnosis and treatment will lose some of their drug efficacy because of the accelerated clearance. Hence, it is of great importance to know whether the ABC phenomenon can be induced by polymeric micelles or not. Recently, the accelerated clearance of [^{3}H]-labeled PEGylated liposomes was observed in mice pre-administered with an empty polymeric micelle composed of poly (ethylene glycol)-*b*-poly (β-benzyl L-aspartate) (PEG-PBLA) 50 nm in diameter (16).

In this study, we first investigated whether the ABC effect was caused by repeated injections of a polymeric micelle encapsulating Gd-DOTA (Gd-micelle) and of a PEGylated liposome encapsulating Gd-DTPA (Gd-liposome) as a positive control. Concentrations of Gd ions were measured for this investigation. Furthermore, we examined the effect of a PEG homopolymer on the tissue distribution of Gd-liposomes.

MATERIALS AND METHODS

Materials

Magnevist® (Gd-DTPA) was purchased from Bayer Schering Pharma (Berlin, Germany). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (mPEG$_{2000}$-DSPE), hydrogenated soy bean phosphatidylcholine (HSPC), and egg phosphatidylcholine (EPC) were purchased from the NOF Corporation (Tokyo, Japan). Cholesterol and polyethylene glycol 500,000 (PEG$_{500,000}$) were of analytical grade (Wako Pure Chemical, Osaka, Japan). All lipids were used without further purification. All other reagents were of analytical grade.

Animals

Four-week-old female ddY mice were purchased from Sankyo Lab Service Corp. (Tokyo, Japan). All care and handling of animals were performed with the approval of the Animal and Ethics Review Committee of Hoshi University and of Principles of Laboratory Animal Care (NIH #publication 85-23, revised in 1985).

Preparation of the Gd-micelle

Synthesis of a chelate moiety-binding block copolymer was performed as reported in our previous paper (19). Briefly, a poly(ethylene glycol)-*b*-poly(L-lysine) block copolymer (PEG-P(Lys)) was prepared through acid hydrolysis of a poly(ethylene glycol)-*b*-poly[ε-(benzyloxycarbonyl)-L-lysine] (PEG-P(Lys(Z)) block copolymer (Fig. 1). We synthesized PEG-P(Lys(Z) with polymerization of a Lys(Z) *N*-carboxy anhydride monomer from PEG-NH_2 (molecular weight of PEG-NH_2 = 5,200). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (*N*-hydroxysuccinimide ester) was fully conjugated to lysine residues of PEG-P(Lys).

The composition of PEG-P(Lys-DOTA) was determined by means of ^{1}H-NMR spectroscopy in D_2O under alkali conditions (pH>10). $GdCl_3 \cdot 6H_2O$ was added to PEG-P(Lys-DOTA) at pH 6.0 to 6.5 for 3 hr at 50°C. Gd content was determined using inductively coupled plasma (ICP) (SPS7800, SII Nano Technology Inc., Tokyo, Japan). We obtained the block copolymer as PEG-P(Lys-DOTA-Gd) (Gd content = 7.7 wt%, the number average of Gd is 8.2). The block copolymer formed a polymeric micelle spontaneously in an aqueous solution (Gd-micelle). The size and zeta-potential of the Gd-micelle diluted with saline for three independent preparations was 84.5±6.0 nm and −1.70±0.80 mV, respectively, at 25°C as determined by dynamic light scattering (ELS-Z2, Otsuka Electronics Co., Ltd., Osaka, Japan).

Preparation of the Empty Liposome and Gd-liposome

First, an empty liposome, which induced the ABC phenomenon, was prepared by the lipid film hydration method as described previously (21). Briefly, a mixture of HSPC, cholesterol, and mPEG$_{2000}$-DSPE in a molar ratio of 1.85:1.0:0.15 was dissolved in chloroform. The solution was

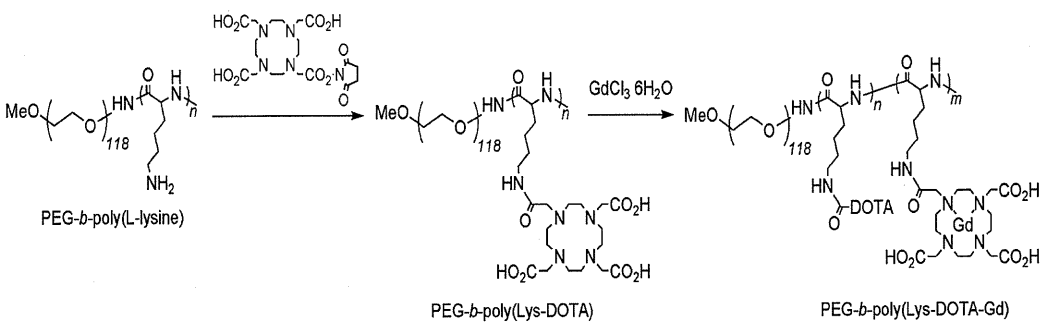

Fig. 1. Synthesis of PEG-P(Lys-DOTA-Gd).

evaporated dry to form the lipid film. Then, the liposome was produced by hydration of the lipid film with saline, followed by size reduction with sonication. The size and zeta-potential of the liposome diluted with saline were 178.5 nm and −22.1 mV, respectively.

Two kinds of Gd-liposomes were prepared because Gd-DTPA content was dependent on the preparation methods. One kind of Gd-liposome was prepared by an ethanol injection method (GdL-E). In brief, a mixture of EPC, cholesterol, and $mPEG_{2000}$-DSPE in a molar ratio of 2.15:0.88:0.15 was dissolved in ethanol and then hydrated with Gd-DTPA at 50°C. The resulting liposomes were sonicated for 10 min, then subjected to exhaustive dialysis against phosphate-buffered saline (PBS, 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4) with a dialysis membrane having a 2,000 molecular-weight cutoff for 24 hr. The size and zeta-potential of the liposome were 150.1±18.8 nm and −0.94±6.78 mV, respectively, for three independent preparations. As a control of GdL-E, an empty liposome not including Gd-DTPA (empty GdL-E) was prepared by the same method as the Gd-liposome (GdL-E), except that saline was used to hydrate the ethanol solution of lipid. The particle size of empty GdL-E was 139.5 nm. Another kind of Gd-liposome was prepared by reverse phase evaporation (GdL-R) to encapsulate a larger amount of Gd-DTPA. The lipid was the same as GdL-E described above and dissolved in 4 mL of chloroform and 2 mL of diethyl ether. Gd-DTPA was added to the lipid solution. The mixture was sonicated to form an emulsion, which was evaporated to produce the liposome. Finally, the resulting liposome was sized at 60°C on an extruder (Avanti Polar Lipids, Inc., AL, USA) with three passes through a 0.4 μm Nuclepore membrane (Waterman, Maidstone, UK) and five passes through a 0.2 μm Nuclepore membrane, followed by exhaustive dialysis as described above. The particle size and zeta-potential of the liposome were 140.9± 13.5 nm and −2.52±5.18 mV, respectively, for three independent preparations. The phospholipid concentration of the liposome including HSPC or EPC was measured with the Phospholipids C-test Wako (Wako Pure Chemical Industries, Ltd.). GdL-E contained 2.26 μmol Gd per 10 μmol lipids, and GdL-R contained 2.29 μmol Gd per 5 μmol lipids.

Release Studies of Gd-micelle and Gd-liposomes

The release of Gd−DTPA from Gd−liposome (GdL−E or GdL−R) and Gd from Gd−micelle was evaluated by dialysis method using a Spectrapor 6 tubing with molecular weight cut-off of 1,000 Da (Spectrum Laboratories Inc., Tokyo, Japan). Briefly, the sample of Gd-micelle containing 1.2 mM Gd and Gd-liposomes of GdL-E containing 0.96 mM Gd-DTPA or GdL-R containing 0.96 mM Gd-DTPA (1 mL) were dialyzed against PBS (pH 7.4, 200 mL) at 37°C. At the indicated time points (10 min, 1, 3, 6, 24 h), 1 mL aliquots of the medium were withdrawn, and the same volume of fresh medium was added. The Gd concentration was analyzed by ICP. The accumulative release of Gd or Gd-DTPA released from the Gd-micelle or Gd-liposome, respectively was expressed as a percentage of the released Gd or Gd-DTPA and plotted as a function of time.

Pharmacokinetics and Tissue Distribution of the Gd-micelle and Gd-liposome

For pharmacokinetics study, the mice were intravenously injected with the Gd-micelle at a dose of 33 μmol Gd/kg (67.3 mg polymer/kg) or the Gd-liposomes including GdL-E at a dose of 6.75 μmol Gd/kg and 10 μmol lipids/kg and GdL-R at 2.65 μmol Gd/kg and 5 μmol lipids/kg. About 30 to 100 μL of blood were taken from a tail vein with a quantitative capillary at 10 min, 1 h, 3 h, 6 h, and 24 h after the injection. The Gd-micelle or the Gd-liposome was injected into a lower part of a tail vein, and blood sample was taken at a certain time point described above from an upper part of the tail vein at the other side of the injected vein. Therefore, this experiment was free from the sample pollution problem. The blood samples were added to saline and centrifuged at 3,000 rpm for 15 min, and the supernatant was used to measure Gd content by ICP. The elimination half-life ($T_{1/2}$) was calculated based on a single compartment model. For the tissue distribution of Gd-micelles and Gd-liposomes study, the second dose of Gd-micelles or Gd-liposomes was injected intravenously through the tail vein at a certain time interval after the first injection. Samples of blood were taken from the hepatic portal vein 6 h after the second injection, and tissues of liver, spleen, and kidney were removed at the same time. The plasma and blood volume were calculated as 0.0488 mL/g body weight for plasma and 0.0778 mL/g body weight for blood, respectively (19).

Measurement of Gd Content

For the quantitative determination of Gd content, blood samples were centrifuged at 3,000 rpm for 15 min, and then plasma was taken out and diluted with 0.1% HNO_3 for ICP. Tissue samples of the liver, spleen, and kidney were digested with a mixture of 98% H_2SO_4 and 62% HNO_3 (1:2, *v:v*) and then subjected to ICP.

Statistical Analysis

The statistical analysis was performed with the Dunnett's multiple comparison test. The level of significance was set at $p<0.05$ or $p<0.01$.

RESULTS

Release Behavior of Gd-micelle and Gd-liposomes

Gd or Gd-DTPA release behavior from Gd-micelle or Gd-liposomes was studied by the dialysis method. As shown in Fig. 2, only 0.2% of Gd leaked from the Gd-micelle at 37°C in PBS (pH 7.4) for 24 h. On the other hand, 4.8% of entrapped Gd-DTPA leaked from the Gd-liposome prepared by reverse phase evaporation method (GdL-R) and 22.4% for 24 h from the Gd-liposome prepared by ethanol injection method (GdL-E). Hence, it is obvious that Gd-micelle has hardly release behavior of Gd, and GdL-R showed much slower release than GdL-E. The results indicated that the leakage of Gd or Gd-DTPA from nanocarriers was greatly affected by the preparation methods.

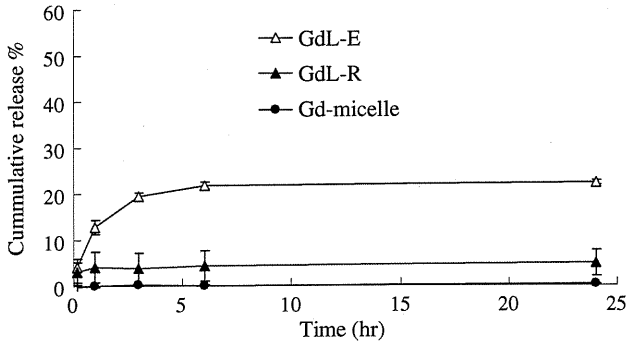

Fig. 2. Release profiles of Gd from Gd-micelle or Gd-DTPA from Gd-liposomes prepared by ethanol injection method (GdL-E) and reverse phase evaporation method (GdL-R) in PBS (pH 7.4) at 37°C. Data represent mean±S.D. (n=3).

Pharmacokinetics of the Gd-micelle and Gd-liposome

As shown in Fig. 3, at 10 min after the intravenous injection, 33.3% of the injected dose was found in blood for the Gd-micelle, and 40.0% and 50.3% for the Gd-liposome of GdL-E and GdL-R, respectively. At a dose of 33 μmol Gd/kg, the $T_{1/2}$ of the Gd-micelle was 10.2±3.9 h. Besides, the $T_{1/2}$ of GdL-E at a dose of 6.75 μmol Gd/kg and GdL-R at a dose of 2.65 μmol Gd/kg were 5.9±0.5 h and 6.0±1.0 h, respectively. In a previous study, we showed that Gd-DTPA was very rapidly cleared from the bloodstream with a minute's order half-life (19). Therefore, the detected Gd in blood is considered to be Gd-DTPA encapsulated in the liposome in a quantitative manner for measurements 6 h post intravenous injection. On the other hand, the main purpose of this study is the ABC phenomenon of a polymeric micelle MRI contrast agent, and PEGylated liposome is used as a positive control for the ABC phenomenon. Therefore, detection of liposome with Gd measurements is appropriate for the present purpose.

Effect of the First Dose on the Distribution of the Gd-micelle

The effects of the first dose on the distribution of the Gd-micelle injected a second time were evaluated. When the second dose of Gd-micelle was fixed at 33 μmol/kg, there was no significant difference of percent injected doses in plasma, kidney, and spleen between various first doses of the Gd-micelle from 0 to 100 μmol/kg (Fig. 4A). A dose of 100 μmol Gd/kg is the clinical dose of Gd-DTPA (17). Interestingly, the distribution of Gd-micelles in plasma, kidney, spleen and liver with the first injection of the empty liposome was similar to that with the first injection of saline. For the liver, the percent injected dose after a first dose of 33 μmol/kg and 100 μmol/kg was significantly higher than in the control saline group, possibly due to the incomplete elimination of the first dose of the Gd-micelle in liver at day 7 because of high doses of polymeric micelles (67.3 mg ~ 203.9 mg polymer/kg). The dose of 2 μmol Gd/kg of the Gd-micelle was the minimum at which Gd was detectable by means of ICP 6 h after injection. As shown in Fig. 4B, when the second dose of the Gd-micelle was decreased to 5 μmol/kg and 2 μmol/kg, the distribution was similar to that of 33 μmol/kg (Fig. 4A). Hence, the results showed that the tissue distribution of the Gd-micelle at the second dose of 33, 5, or 2 μmol/kg was not affected significantly except in liver by pre-administration of the Gd-micelle or the empty liposome. Although Gd in the first dose may interfere with the Gd accumulation in liver following the second dose injection, Gd-micelle as the first dose for micelle-forming properties are

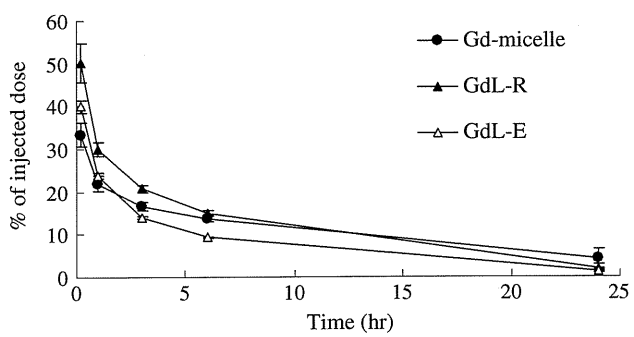

Fig. 3. Plasma elimination profiles of Gd following a single intravenous injection of Gd-micelle at a dose of 33 μmol Gd/kg and Gd-liposome including Gd-liposome prepared by ethanol injection method (GdL-E) at a dose of 6.75 μmol Gd/kg and Gd-liposome prepared by reverse phase evaporation method (GdL-R) at a dose of 2.65 μmol Gd/kg. Data represent mean±S.D. (n=3–4).

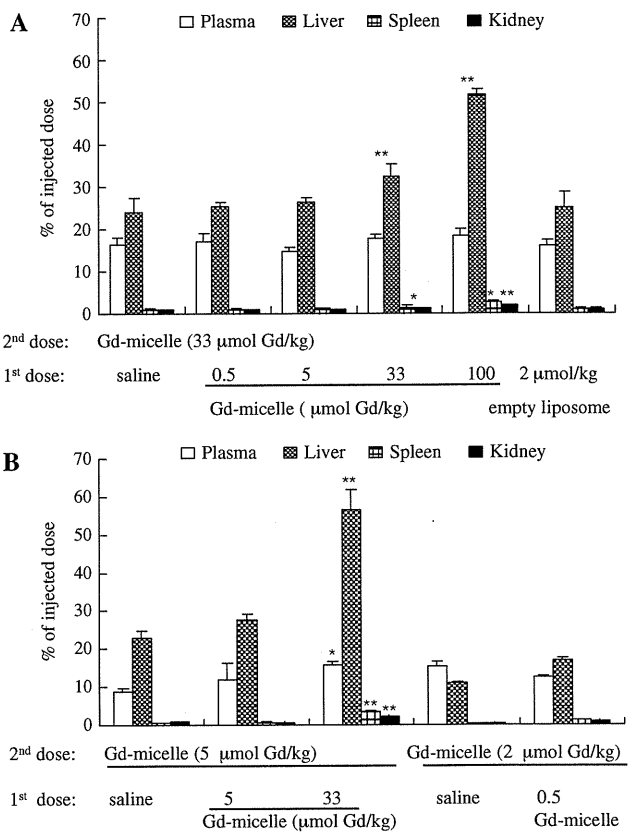

Fig. 4. Effect of the first dose on the tissue distribution of Gd-micelle. The second dose of Gd-micelle with 33 μmol/kg (**A**) or 5 μmol/kg or 2 μmol/kg (**B**) was intravenously injected at day 7 after the first injection of 0.5, 5, 33, 100 μmol/kg of Gd-micelle or the empty liposome at a dose of 2 μmol lipid/kg. Tissues including blood, liver, spleen, and kidney were taken out 6 h after the second injection of Gd-micelle. Data represent mean±S.D. (n=3, 6). P values apply to differences between the saline group and Gd-micelle or liposome treated group. *p<0.05, **p<0.01.

Gd-content-dependent, and Gd-free polymeric micelle is different from the Gd-containing micelle in size and micelle forming characteristics.

Effect of Time Interval Between the Two Injections on the Distribution of the Gd-micelle

Since it was reported that the ABC effect was maximized when the interval between the two injections of liposome was 10 days in mice (7,12), we changed the time interval for the injection of Gd-micelle at a dose of 33 μmol/kg from 3 days to 10 days. No significant difference in plasma Gd levels (15 ~ 18% dose) was observed between the control group and the groups with different time intervals 6 h after the second injection (Fig. 5). The control group was given the Gd-micelle at 33 μmol/kg after a first injection of saline. The Gd% of injected dose in the liver was much higher at day 3 after the second injection than that on other days, probably due to the incomplete elimination of the first dose of the Gd-micelle.

Effect of the First Dose on the Distribution of the Gd-liposome

Since a lower dose of lipid in the first injection results in a more significant ABC, the effects of dose were investigated. The first dose of the Gd-micelle (5 μmol Gd/kg), empty GdL-E (2 μmol lipids/kg), or GdL-E (2 μmol lipids/kg corresponding to 0.45 μmol Gd/kg) was given with a second dose of GdL-E at 10 μmol lipids/kg corresponding to 2.26 μmol Gd/kg. As shown in Fig. 6, the first injection of the Gd-micelle resulted in a similar percentage of the injected dose of the Gd-liposome in plasma, liver, spleen, and kidney in comparison with the saline group. On the other hand, after the second injection of GdL-E, the Gd concentrations in plasma and kidney were too low to be detected, with the first injection of the empty GdL-E and the GdL-E. At that time, the %dose in the liver significantly increased, but that in spleen significantly decreased as compared to saline ($p<0.05$).

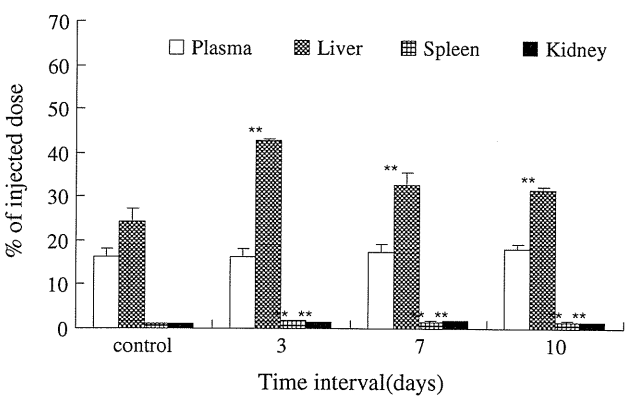

Fig. 5. Effect of the time intervals on the tissue distribution of Gd-micelle. The second dose of Gd-micelle at 33 μmol/kg was intravenously injected at day 3, day 7, or day 10 after the first injection of the same micelle at 33 μmol/kg. The control group was referred to the second dose of Gd-micelle at a dose of 33 μmol/kg with the first injection of saline. Tissues including blood, liver, spleen, and kidney were taken out at 6 h after the second injection of Gd-micelle. Data represent mean±S.D. ($n=3$). *P* values apply to differences between the control group and treated group. $*p<0.05$, $**p<0.01$.

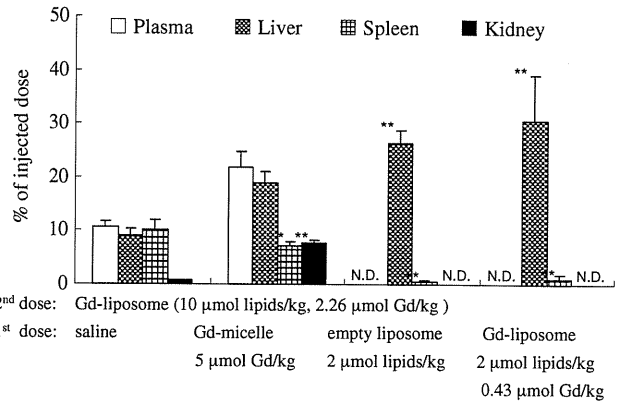

Fig. 6. Effect of the first dose on the tissue distribution of Gd-liposome (GdL-E). The second dose of GdL-E with 10 μmol lipids/kg and 2.26 μmol Gd/kg was intravenously injected at day 7 after the first injection of Gd-micelle (5 μmol Gd/kg), empty GdL-E (2 μmol lipids/kg), and GdL-E (2 μmol lipids/kg and 0.43 μmol Gd/kg). Tissues of blood, liver, spleen, and kidney were removed 6 h after the second injection of GdL-E liposome. Data represent mean±S.D. ($n=3$). *P* values apply to differences between the saline group and Gd-micelle or liposome treated group. $*p<0.05$, $**p<0.01$. N.D. The Gd concentration was too low to be detected by ICP.

Therefore, the data herein show that the accelerated clearance of Gd-liposome at 10 μmol lipids/kg corresponding to 2.26 μmol Gd/kg was induced by both the Gd-liposome and empty liposome, but not by the Gd-micelle. This finding indicates that Gd ions at the first dose of 0.45 μmol/kg did not affect the induction of ABC caused by liposomes.

Effect of PEG on the Distribution of Gd-liposomes

Next, the effect of injecting a PEG homopolymer and PEG_{2000}-DSPE on the distribution of Gd-liposomes was examined. Since the encapsulation efficiency of Gd was low with the ethanol injection method, we prepared another Gd-liposome by the reverse phase evaporation method (GdL-R). The tissue distribution of GdL-R at 6 h after injection at a dose of 5 μmol lipids/kg was not significantly influenced by the pre-administration of 50 mg/kg PEG $_{500,000}$, 0.2 mg/kg PEG $_{500,000}$, or 0.3 mg/kg PEG_{2000}-DSPE 7 days before (Fig. 7). The dose of 0.2 mg/kg PEG $_{500,000}$ and 0.3 mg/kg PEG_{2000}-DSPE with the concentration of 0.04 mg/ml is similar to that of the 5 mol% PEGylated liposome (0.3 mg/kg PEG_{2000}-DSPE), which could produce the ABC phenomenon (Fig. 6). Hence, the first injection of $PEG_{500,000}$ saline or PEG_{2000}-DSPE saline failed to cause the ABC phenomenon after the second administration of Gd-liposome. Hence, only injections of PEG macromolecules did not induce the ABC effect.

DISCUSSION

In the present study, the influence of dose on the tissue distribution of Gd-micelles after repeated administrations was investigated. Many studies have found that a lower dose of lipid in liposomes or nanoparticles results in a greater ABC effect (6–8,12), and the magnitude of the ABC phenomenon reached a maximum when the time interval between two

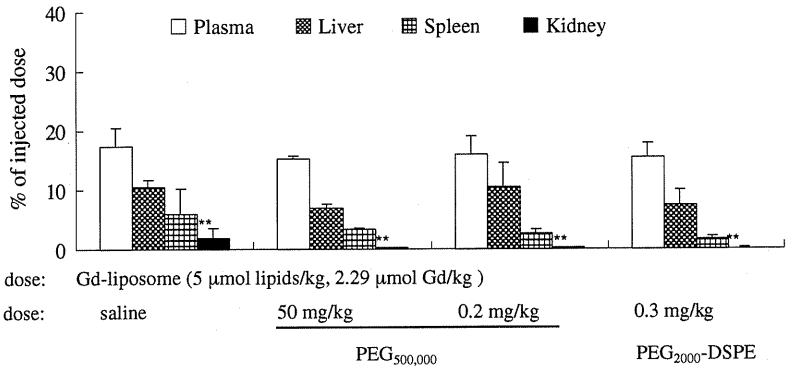

Fig. 7. Effect of $PEG_{500,000}$ and PEG_{2000}-DSPE on the tissue distribution of Gd-liposome (GdL-R). The second dose of Gd-liposome with 5 μmol lipids/kg and 2.29 μmol Gd/kg was intravenously injected at day 7 after the first injection of $PEG_{500,000}$ saline at a dose of 50 mg/kg or 0.2 mg/kg, and PEG_{2000}-DSPE saline at 0.3 mg/kg. The control group of GdL-R was injected at a dose of 10 μmol lipids/kg with the first injection of saline. Tissues of blood, liver, spleen, and kidney were removed at 6 h after the second injection of GdL-R. Data represent mean±S.D. (n=3–5). P values apply to differences between the saline group and $PEG_{500,000}$ or PEG_{2000}-DSPE treated group. *p<0.05, **p<0.01.

injections was 5–7 days in rats (5) and 10 days in mice (7,12). Hence, we investigated the distribution of the Gd-micelle at various doses and an interval of 3, 7, or 10 days between injections. We found that repeated injections of the Gd-micelle, even with the second dose reduced to 2 μmol Gd/kg (corresponding to 4 mg polymer/kg) and at different time intervals at a dose of 33 μmol Gd/kg (corresponding to 67.3 mg polymer/kg), did not result in an accelerated clearance. ABC of the second injection of the Gd-liposome was induced by the first injection of both the Gd-liposome and the empty PEGylated liposome, but not by the first injection of the Gd-micelle (Fig. 6), Gd-DTPA encapsulated in liposomes would not affect the ABC phenomenon. Therefore, our observation that the ABC phenomenon did not occur with the Gd-micelle is important as it means that injections of the Gd-micelle will not change the biodistribution of a second administration of diagnostic or therapeutic agents.

For Gd-micelle, Gd was chelated to the micelle and thus existed in the form of micelle as shown in Fig. 1, which was consistent with the release results in Fig. 2 that Gd did not leak from the Gd-micelle in PBS (pH 7.4). Gd concentration in plasma, therefore, will reflect the pharmacokinetic behavior of the Gd-micelle. For Gd-liposomes, although the Gd concentration in plasma contained both the leaked Gd-DTPA from the Gd-liposome and the encapsulated Gd-DTPA in the Gd-liposome, the leaked-free Gd-DTPA is reported to be very rapidly cleared from the bloodstream with a minute's order half-life (19), and thus the detected Gd in blood is considered to be only the Gd-DTPA encapsulated in the liposome 6 h after intravenous injection in this study. Compared to GdL-R, the leakage of Gd-DTPA from GdL-E was faster, resulting in the lower Gd concentration (the encapsulated Gd) of GdL-E in blood in Fig. 3. Most importantly, the purpose of this study is to investigate if the distribution for the second dose of the Gd-liposomes or Gd-micelle was affected after pre-administered with the first dose or not. Therefore, the leakage of Gd-DTPA from the Gd-liposomes will not influence this study. In addition, many studies demonstrated that ABC phenomenon for empty liposome was observed determined by [H^3]-labelled or ^{99m}Tc-labelled method (5–9).

It is believed that macrophages in the RES play an important role in ABC, and liposomes were mainly located in Kupffer cells after a second injection (5,8).When hepatosplenic macrophages were depleted, no enhanced clearance of liposomes was observed (6). The induction of ABC with liposomes could be attributable to a 150 kDa serum factor (5), anti-PEG IgM (9,11,12,22,23), anti-PEG antibody (10), or anti-PEG IgG antibody (24).

Whereas the mechanism of the immune response on repeated injections of liposomes has not been fully elucidated yet, the enhanced clearance effect can still be divided into two phases: the induction phase following the first injection and the effectuation phase following the second injection (6). According to this theory, there are two very important factors: one is the biological material (e.g. antibody) produced in the induction phase, the other is the recognition of the antibody by the second dose. For the effectuation phase, it was reported that the ABC phenomenon was induced by the second dose of a PEGylated liposome, but not of a liposome lacking a PEG-coating (23). This indicates that PEG is essential for the nanocarrier to recognize the antibody in the effectuation phase. In this study, the ABC phenomenon was not observed after repeated injections of the Gd-micelle at different doses and time intervals. This ABC failure of Gd-micelle may be caused by the failure for the production of biological material in the induction phase (data not shown) or/and for the recognization by the antibody in the effectuation phase. Even if the first injection was of empty liposome, the second injection of the Gd-micelle did not produce the ABC phenomenon either. This suggests that the antibody produced by the empty liposome in the induction phase is not recognized by the PEG moiety of the Gd-micelle. Therefore, not only PEG, but also other factors such as structure and hydrophobic character affect recognition.

For the induction phase, the ABC phenomenon was not observed when the amount of PEGylated lipid of liposomes in the first injection was more than 10 mol% (7,8). We have previously reported the accelerated clearance of [^{3}H]-labeled PEGylated liposomes in mice pre-administered empty PEG-PBLA polymeric micelles (16). Furthermore, repeated

injections of PEG-PLA nanoparticles also produced the ABC phenomenon (12). Hence, the structure and component of nanocarriers has a considerable impact on the induction phase of ABC. From a structural perspective, the Gd-micelle formed through ionic interactions; therefore, it does not have any hydrophobic part (Fig. 1). In contrast, the PEG-PBLA micelle is composed of both a hydrophilic part, PEG, and a hydrophobic part, PBLA. Similarly, PEGylated liposomes possess a hydrophilic PEG chain and a hydrophobic bilayer membrane. The immunogenicity of an antigen can be affected by factors such as the physical and chemical properties of the antigen, its dose, and so on (25). The reasons why the Gd-micelle evaded the ABC phenomenon have not yet been elucidated at the present stage. The absence of a hydrophobic part may be a key for this elucidation because the other ABC-phenomenon-positive PEG-based carrier systems possess hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. We are currently investigating the ABC phenomenon induced by other kinds of polymeric micelles and nanoparticles. It is hoped that these experiments will provide more evidence for the mechanism of the ABC phenomenon.

CONCLUSIONS

The Gd-micelle did not induce ABC following its pre-administration at various doses and time intervals. In contrast, the Gd-liposome induced the phenomenon when it or an empty PEGylated liposome, but not the $PEG_{500,000}$ macromolecule or PEG_{2000}-DSPE, was pre-administered. ABC-phenomenon-positive PEG-based carrier systems possess a hydrophobic part in a hydrophobic inner core for polymeric micelles and in a lipid bilayer for PEG-liposomes. The absence of a hydrophobic part of Gd-micelle may be a key factor for not producing the ABC phenomenon.

ACKNOWLEDGMENTS

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REFERENCES

1. Allen TM, Hansen C, Martin F, Redemann C, Yau-Young A. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives *in vivo*. Biochim Biophys Acta. 1991;1066:29–36.
2. Woodle MC, Lasic DD. Sterically stabilized liposomes. Biochim Biophys Acta. 1992;1113:171–99.
3. Lasic DD, Martin FJ, Gabizon A, Huang SK, Papahadjopoulos D. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim Biophys Acta. 1991;1070:187–92.
4. Torchilin VP, Omelyanenko VG, Papisov MI, Bogdanov AA Jr, Trubetskoy VS, Herron JN, *et al.* Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity. Biochim Biophys Acta. 1994;1195:11–20.
5. Dams ET, Laverman P, Oyen WJ, Storm G, Scherphof GL, van Der Meer JW, *et al.* Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. J Pharmacol Exp Ther. 2000;292:1071–9.
6. Laverman P, Carstens MG, Boerman OC, Dams ET, Oyen WJ, van Rooijen N, *et al.* Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. J Pharmacol Exp Ther. 2001;298:607–12.
7. Ishida T, Ichikawa T, Ichihara M, Sadzuka Y, Kiwada H. Effect of the physicochemical properties of initially injected liposomes on the clearance of subsequently injected PEGylated liposomes in mice. J Control Release. 2004;95:403–12.
8. Ishida T, Harada M, Wang XY, Ichihara M, Irimura K, Kiwada H. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. J Control Release. 2005;105:305–17.
9. Ishida T, Kiwada H. Accelerated blood clearance (ABC) phenomenon upon repeated injection of PEGylated liposomes. Int J Pharm. 2008;354:56–62.
10. Judge A, McClintock K, Phelps JR, Maclachlan I. Hypersensitivity and loss of disease site targeting caused by antibody responses to PEGylated liposomes. Mol Ther. 2006;13:328–37.
11. Semple SC, Harasym TO, Clow KA, Ansell SM, Klimuk SK, Hope MJ. Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol–lipid conjugates and nucleic acid. J Pharmacol Exp Ther. 2005;312:1020–6.
12. Lu W, Wan J, She ZJ, Jiang XG. Brain delivery property and accelerated blood clearance of cationic albumin conjugated pegylated nanoparticle. J Control Release. 2007;118:38–53.
13. Gabizon A, Isacson R, Rosengarten O, Tzemach D, Shmeeda H, Sapir R. An open-label study to evaluate dose and cycle dependence of the pharmacokinetics of pegylated liposomal doxorubicin. Cancer Chemother Pharmacol. 2008;61:695–702.
14. Kakizawa Y, Kataoka K. Block copolymer micelles for delivery of gene and related compounds. Adv Drug Deliv Rev. 2002;54:203–22.
15. Yokoyama M, Okano T, Sakurai Y, Ekimoto H, Shibazaki C, Kataoka K. Toxicity and antitumor activity against solid tumors of micelle-forming polymeric anticancer drug and its extremely long circulation in blood. Cancer Res. 1991;51:3229–36.
16. Koide H, Asai T, Hatanaka K, Urakami T, Ishii T, Kenjo E, *et al.* Particle size-dependent triggering of accelerated blood clearance phenomenon. Int J Pharm. 2008;362:197–200.
17. Lin SP, Brown JJ. MR contrast agents: physical and pharmacologic basics. J Magn Reson Imaging. 2007;25:884–99.
18. Mulder WJ, Strijkers GJ, van Tilborg GA, Griffioen AW, Nicolay K. Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. NMR Biomed. 2006;19:142–64.
19. Shiraishi K, Kawano K, Minowa T, Maitani Y, Yokoyama M. Preparation and in vivo imaging of PEG-poly(L-lysine)-based polymeric micelle MRI contrast agents. J Control Release. 2009;136:14–20.
20. Nakamura E, Makino K, Okano T, Yamamoto T, Yokoyama M. A polymeric micelle MRI contrast agent with changeable relaxivity. J Control Release. 2006;114:325–33.
21. Wang XY, Ishida T, Ichihara M, Kiwada H. Influence of the physicochemical properties of liposomes on the accelerated blood clearance phenomenon in rats. J Control Release. 2005;104:91–102.
22. Ishida T, Ichihara M, Wang XY, Yamamoto K, Kimura J, Majima E, *et al.* Injection of PEGylated liposomes in rats elicits PEG-specific IgM, which is responsible for rapid elimination of a second dose of PEGylated liposomes. J Control Release. 2006;112:15–25.
23. Wang XY, Ishida T, Kiwada H. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. J Control Release. 2007;119:236–44.
24. Sroda K, Rydlewski J, Langner M, Kozubek A, Grzybek M, Sikorski AF. Repeated injections of PEG-PE liposomes generate anti-PEG antibodies. Cell Mol Biol Lett. 2005;10:37–47.
25. Abbas AK, Lichtman AH, Pober JS. Cellular and molecular immunology. Philadelphia: Saunder; 1991.

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Polymeric micelles as a new drug carrier system and their required considerations for clinical trials

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Importance of the field: A polymeric micelle is a macromolecular assembly composed of an inner core and an outer shell, and most typically is formed from block copolymers. In the last two decades, polymeric micelles have been actively studied as a new type of drug carrier system, in particular for drug targeting of anticancer drugs to solid tumors.

Areas covered in this review: In this review, polymeric micelle drug carrier systems are discussed with a focus on toxicities of the polymeric micelle carrier systems and on pharmacological activities of the block copolymers. In the first section, the importance of the above-mentioned evaluation of these properties is explained, as this importance does not seem to be well recognized compared with the importance of targeting and enhanced pharmacological activity of drugs, particularly in the basic studies. Then, designs, types and classifications of the polymeric micelle system are briefly summarized and explained, followed by a detailed discussion regarding several examples of polymeric micelle carrier systems.

What the reader will gain: Readers will gain a strategy of drug delivery with polymeric carriers as well as recent progress of the polymeric micelle carrier systems in their basic studies and clinical trials.

Take home message: The purpose of this review is to achieve tight connections between the basic studies and clinical trials.

Keywords: anticancer drug, block copolymer, EPR effect, polymeric micelle, targeting, toxicity

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1. Introduction

Polymeric micelles have lately appeared as a new type of drug carrier; the preceding drug carriers were micro(nano)particles, liposomes and non-micelle-forming polymeric carriers. The study of polymeric micelle drug carriers started in the 1980s [1-4], and these carrier systems were recognized as one of the most potent drug carrier types in the 1990s [5-13]. Then, in the 2000s, several significant related clinical trials [14-23] got underway, while more and more R&D projects were conducted.

A polymeric micelle is a macromolecular assembly composed of an inner core and an outer shell. The polymeric micelles can have a spherical or a cylindrical shape, depending on the chemical structure and chain length of the macromolecules. For the purpose of drug targeting, most polymeric micelle studies have dealt with the spherical shape, whereas a very limited number of the filamentous shape systems have been studied [24,25]. Therefore, this review deals only with the spherical polymeric micelles. A spherical polymeric micelle structure forms from block copolymers or graft copolymers [26]. **Figure 1** illustrates the formation of a polymeric micelle structure resting on an AB-type block copolymer in which two polymer chains are of a tandem-connected form. Drug molecules are incorporated into the inner core of the micelle through both chemical conjugation and physical

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Article highlights.

- In this review, I discuss toxicities of the polymeric micelle carrier systems as well as pharmacological activities of the block copolymers insofar as these subjects have not been well discussed irrespective of their importance in clinical applications.
- There are two types of drug incorporation into the inner core of the polymeric micelles, namely chemical conjucation and physical entrapment.
- Other than targeting, three functions can be obtained with the polymeric micelles.
- In the system in Kabanov's report, the block copolymer worked as a biologically active agent rather than as a carrier to specific sites.
- In the limited situation, the research team reported on three important observations, which merit an explanation and a discussion here.
- There are six widely known clinical trials regarding polymeric micells drug carrier systems.

This box summarises key points contained in the article.

entrapment. Such a micellar structure forms if one segment of the block copolymer can provide enough interchain cohesive interactions in a selective solvent. For the cohesive interactions, hydrophobic interactions have been used most because most drug molecules possess a hydrophobic character. Typically, the polymeric studies on drug carriers have involved AB- or ABA-type block copolymers because the close relationship between micelle-forming behavior and the structure of polymers can be evaluated more easily with these types of block copolymer than with the other types.

In this review, toxicities of the polymeric micelle carrier systems are discussed as well as pharmacological activities of the block copolymers insofar as these subjects have not been well discussed irrespective of their importance in clinical applications. (Please see other excellent reviews [27-32] for more general information on the polymeric micelle drug carrier systems.) In this explanation of these toxicity and activity subjects, anticancer targeting systems are referenced because most studies on polymeric micelle drug carrier systems have concerned anticancer targeting to solid tumors. **Figure 2** summarizes the developmental process of drug targeting systems, which has comprised the basic study stage, the preclinical study stage and the clinical trial stage. The basic study includes syntheses of polymers, preparation of polymeric micelles containing drugs, *in vitro* cytotoxicity tests, *in vivo* anticancer activity tests and *in vivo* pharmacokinetic analyses. The basic study includes *in vivo* toxicity tests, but these tests span a narrower examination range than do preclinical tests. In the preclinical tests, anticancer activity is examined with reference to a greater variety of cancer models, and pharmacokinetic analyses are carried out in greater detail than is the case of the basic study. Furthermore, in the preclinical stage, toxicities undergo detailed examination relative to various kinds of animals. Clinical trials are carried out in three phases (from Phase I to Phase III). In Phase I trials, the two chief objectives concern toxicity evaluation and determination of a recommended dose for the Phase II trials.

I would argue that, among these three stages, there are slight but significant differences in the importance of evaluations. As illustrated in **Figure 2**, the importance of anticancer activity tests is greater in the basic study than in the preclinical test. On the other hand, the importance of toxicity tests is lower in the basic study than in the preclinical and clinical tests. These differences reflect the purposes and functions of each stage, but the importance of the toxicity evaluations in the basic studies should be stressed, in particular for researchers in the basic sciences. The two overall reasons for emphasizing this point are as follows.

(1) The critical-factor difference between the basic study and the clinical trial. In basic studies, *in vivo* toxicity is assayed at least in anticancer activity tests because high anticancer activity is obtained at the maximum tolerated dose that does not provide lethal toxicity to experimental animals. In this circumstance, one or only a few types of toxicity are critical owing to homogeneity of the experimental animals. Inhibition of the critical toxicity(ies) can be a good strategy for the anticancer activity enhancement. Therefore, basic study researchers tend to pay attention only to one or a very small number of toxicities that are correlated to the maximum tolerated dose. By contrast, many more types of toxicity must be seriously considered and observed in the clinical trials because of the heterogeneity of human patients. A certain type of toxicity that is very mild in the basic study can be critically toxic to some patients owing to genetic characteristics or to a slight disorder in some functions of the patients as well as to physiological differences between the experimental animals and the human beings. If a proportion of the patients receiving the critical (lethal) toxicity is high (e.g., 10%), a drug is dropped from the clinical test, even for anticancer drug cases.

(2) A drug carrier system shows unexpected toxicity in the clinical tests. The unexpected toxicity of an approved drug carrier system is a well-known phenomenon. A liposomal anticancer drug targeting system containing doxorubicin [33,34] was approved in 1995 against Kaposi's sarcoma, followed by FDA approval for treating ovarian cancer. This liposomal system can be targeted to solid tumors owing to its successful poly(ethylene glycol) surface modification, which allows this liposome to use the enhanced permeability and retention effect (EPR effect) [35-37]. The EPR effect is a passive targeting mechanism based on hyperpermeability of the tumor vasculature. One of the most serious adverse effects of this liposome in human clinics is hand-foot syndrome, in which there are observable developed palmar-plantar skin eruptions on the hands and the feet [38,39]. This hand-foot syndrome is never observed with doxorubicin alone or with this liposome carrier alone. This correlation means that the

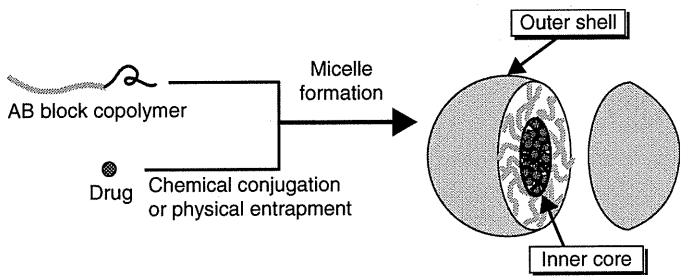

Figure 1. Design of polymeric micelle drug carrier system.

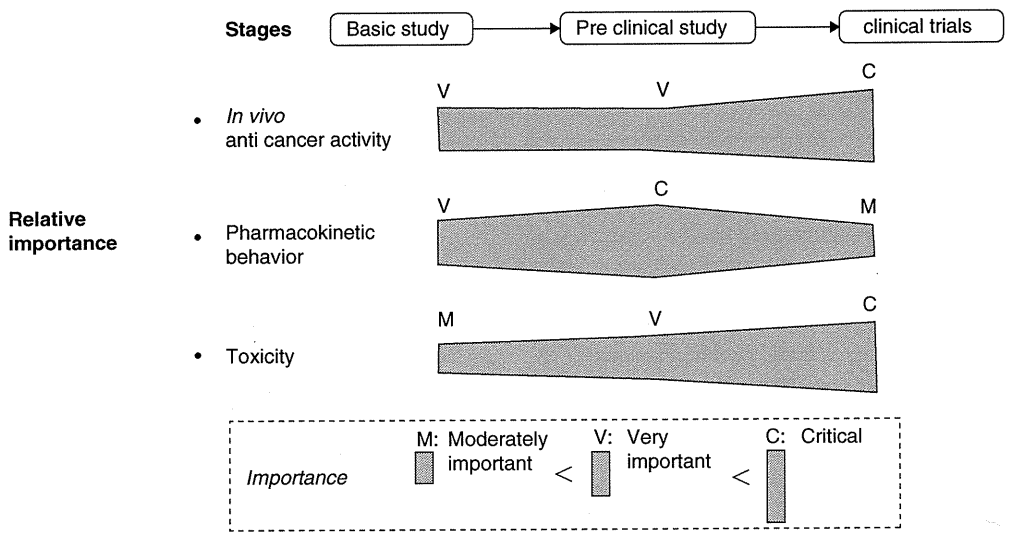

Figure 2. Importance of evaluations on each developmental stage.

adverse effect is observed only in this liposomal doxorubicin carrier system. Furthermore, it is generally believed that delivery to skin tissue is well inhibited for the carriers showing the EPR effect. This hand-foot syndrome was not reported in basic studies of this liposomal doxorubicin; at least, the hand-foot syndrome was not a dose-limiting toxic side effect in mouse anticancer activity assays. Therefore, the appearance of the hand-foot syndrome as a severe side effect in clinical trials was totally unexpected. If skin toxicity had undergone a detailed examination, the hand-foot syndrome could have been reported even in basic studies using mouse models. However, such detailed examinations cannot be conducted in either the basic studies or the preclinical studies for less important side effects surfacing in the mouse models unless some special information related to this toxic effect is present.

This story about the liposomal doxorubicin system does not lead to a necessary conclusion that there should be detailed examinations for all types of toxicity, including unexpected ones. A powerful lesson from the liposomal doxorubicin story is that the system constitutes a good strategy by which basic researchers can pay attention to mild but characteristic side effects in the basic studies.

Drawing on the above-mentioned facts and viewpoints, in this review toxicities of the polymeric micelle targeting systems in animal experiments are summarized and discussed, particularly non-dose-limiting toxicities (i.e., mild toxicities) both of the carrier systems and of the carrier block copolymers.

2. Characterizations and classifications of polymeric micelle drug carrier systems

Before proceeding to the topics in Section 3, which presents some examples of polymeric micelle drug carrier systems, the polymeric micelle systems will be classified depending on their purposes and functions relative to drug delivery system drug delivery system (DDS). This classification should facilitate readers' grasp of the materials in later chapters. Furthermore, the advantages and characteristics of the polymeric micelle are briefly summarized.

2.1 Types of drug incorporation

There are two types of drug incorporation into the inner core of the polymeric micelles, namely chemical conjugation and physical entrapment. Physical entrapment using hydrophobic interactions can be applied to many drugs [40-42], as most drug molecules possess a hydrophobic moiety(ies) (even in the case of water-soluble drugs), and because functional groups that are required for chemical conjugation are not necessary for physical entrapment. In this type of polymeric micelle system, toxicities of carrier block copolymers need not be considered seriously because block copolymers without chemically conjugated drug molecules are expected to be biologically inactive or much less active than the incorporated drug. This type of system can be applied well, particularly to anticancer drug carrier systems because toxicities or pharmacological activities of non-drug-binding block copolymers are believed to be much less intense than those of highly cytotoxic anticancer drugs.

The second type of drug incorporation, chemical conjugation, occurs through chemical bond formation between a drug molecule and the inner-core-forming block of the block copolymer [43-53]. In this type, drug release by cleavage of a chemical bond is an important issue in most systems. One common reaction for this cleavage is hydrolysis. Owing to the phase separation of the inner core from both the outer shell and an outer environment, the drug's access to water molecules, hydrogen ions, hydroxyl ions and hydrolytic enzymes is considerably inhibited. Therefore, the cleavage rate is expected to be much lower than occurs with conventional polymer–drug conjugates. In turn, micelle structures can facilitate the quick release of a drug if the hydrophobicity of the bound drug contributes to inner core association for micelle formation. As the drug molecules are released, the drug release can be accelerated owing to a decrease in inner core hydrophobicity. From this point of view, Li and Kwon [50] have designed methotrexate-conjugated block copolymer micelles, in which the drug release rate can be controlled with conjugated drug amounts. The other possible strategy for chemical conjugation is an alternative action mechanism for the drugs. In a physically entrapped system, only released drugs are expected to express pharmacological activity, even though the delivery systems change both the whole body's distribution and the intracellular distribution. In the case of chemical conjugation, a drug may show activity in both a released form and in a conjugated form. If the conjugated form can express activity, the chemical conjugation systems may overcome the multi-drug resistance induced by P-glycoprotein, as the P-glycoprotein is not expected to result in an efflux of the polymer–drug conjugates to the cells' exteriors. Regarding toxicity concerns, the chemically conjugated drug may pose a risk of a new type of toxicity that was not observed in the corresponding free drug, as the former drug's pharmacokinetic and pharmacodynamic behaviors can be very different from those of the free drug.

2.2 Functions of drug carriers

One important function of the polymeric micelle drug carriers is targeting. As described in Section 2.3, the polymeric micelles possess an inherent ability to target solid tumors owing to the micelles' appropriate size for the EPR effect, which is a passive targeting mechanism to solid tumors.

Other than targeting, three functions can be obtained with the polymeric micelles. The first function is controlled release of a drug. Timing and duration of drug actions can be controlled by controlling drug-release rates from the micelles [51,52,54,55]. This function is not distinctly recognized because both targeting and controlled release functions are achieved in one polymeric micelle system in many cases. In these cases, it is more difficult to prove the contribution of the controlled release for better therapeutic effects than to prove the targeting contribution.

The second function is pharmacological activities of the carrier polymers. In most polymeric micelle systems, block copolymers work only as carriers without showing pharmacological activities. Alternatively, some block copolymers show pharmacological activities. In the former case, toxicity concerns of the carrier block copolymers are not as serious as in the latter case.

There are two types of pharmacologically active copolymer. The first type is the case where block copolymers have a biological activity. Kabanov *et al.* reported that poly(propylene oxide)-*b*-poly(ethylene oxide) block copolymers had an inhibitory activity on P-glycoproteins that play an important role in multi-drug resistance [8,9,19,68,79,70]. Section 3.1 introduces this topic in detail. The second type is a case where drug molecules are attached to the block copolymers. This type of system may have an action mechanism completely different from that of the corresponding free drug (e.g., a cytotoxic action in the conjugate form without a need for the drug release). Such an unusual action mechanism could help result in better pharmacological effects in such matters as circumvention of P-glycoprotein-driven multi-drug resistance. In turn, the new action mechanism has another potential: to cause new toxic side effects that are not found in the corresponding free drug.

The third function of the polymeric micelle systems is solubilization of water-insoluble drugs. Water-insoluble or hardly soluble drugs cannot be safely injected into the bloodstream. One method to overcome this problem is the use of solubilizers that transform these insoluble drugs into a pseudo-soluble status. Water-miscible organic solvents and low-molecular-mass surfactants are representative solubilizers for this purpose. However, these solubilizers are frequently observed showing very high toxicity. Therefore, a DDS carrier that simply solubilizes a water-insoluble drug without any other function is of great value in chemotherapy. Several studies reported that polymeric micelle carriers showed high functions for the solubilization [13,56-62]. This desired function is discussed in Section 2.3, and examples of the solubilization are explained in Sections 3.2 and 3.3.

2.3 Advantages and disadvantages of polymeric micelle carrier systems

Advantages and disadvantages of the polymeric micelle systems as drug carriers are summarized and discussed briefly in this section. Table 1 summarizes the advantages.

The first advantage is the very small size of polymeric micelles. Polymeric micelles are formed typically in a diameter range from 10 to 100 nm, with a substantial narrow distribution. This size range of particles is considered preferable for the attainment of stable, long-term circulation of the carrier system in the bloodstream because the larger particles are actively captured in the reticuloendothelial system and because the smaller particles are rapidly excreted from the kidneys. Alternatively, the small size of polymeric micelles is a great benefit in the sterilization processes associated with pharmaceutical production. Polymeric micelles are easily and inexpensively sterilized by filtration, itself resting on the use of typical sterilization filters with 0.45 or 0.22 μm pores.

The second advantage is the high structural stability of polymeric micelles as compared with the micelles forming from low-molecular-mass surfactants. The high structural stability of polymeric micelles stated above is an important key to *in vivo* delivery in micellar forms (not in single polymer chain form).

The third advantage is the high water solubility of the polymeric micelle drug carrier system incorporating a large amount of hydrophobic drugs [63]. Generally, in conventional polymer–drug conjugate systems, a loss of the water solubility of the polymeric carrier resulting from the introduction of a hydrophobic drug creates a serious problem. Several research groups reported this problem of the drug–polymer conjugates in syntheses [64-66] and in their intravenous injections [67]. Polymeric micelles can incorporate a large number of hydrophobic drug molecules in the large volume of the micelles' inner core, and simultaneously the micelles can maintain their water solubility by inhibiting the intermicellar aggregation of the hydrophobic cores and by using a hydrophilic outer shell layer that works as a barrier against the intermicellar aggregation.

The beneficial character of low toxicity may be described as the fourth advantage. Generally, polymeric surfactants are known to be less toxic than low-molecular-mass surfactants such as sodium dodecyl sulfate. Furthermore, in theory polymeric micelles are considered to be very safe in relation to chronic toxicity. Possessing a much larger size than critical filtration values in the kidney, polymeric micelles can evade renal filtration, even if the molecular mass of the constituting block copolymer is lower than the critical molecular mass for renal filtration. Of course, potential toxicities of polymers and degradation products of polymers (for biodegradable polymers, for example, inflammation induction due to acidic products from polyesters) must be considered. On the other hand, all polymer chains can be released (as single polymer chains) from the micelles during a long time period. This phenomenon results in the complete excretion of the block copolymers from the renal route if the polymer chains are designed with a lower molecular mass than the critical value for renal filtration. Such a result constitutes an advantage of polymeric micelles over the conventional (non-micelle forming) and non-biodegradable polymeric drug carrier systems.

The fifth advantage is separated functionality. Polymeric micelles are composed of two phases: inner core and outer shell. Various functions required for drug delivery systems can be shared by these structurally separated phases. Each phase can play different roles in drug delivery. The outer shell is responsible for interactions with biocomponents such as proteins and cells. These interactions determine pharmacokinetic behavior and the biodistribution of drugs; therefore, the *in vivo* delivery of drugs may be controlled by the outer shell segment independently of the inner core, which is responsible for pharmacological activities through drug loading and release. This heterogeneous structure is more favorable in the construction of highly functionalized carrier systems than in the conventional (non-micelle-forming) polymeric carrier systems, as properties of both phases are freely and independently controlled through a selection of the polymer chains that are appropriate for each segment of block copolymers.

Here, the disadvantages of the polymeric micelle systems will be explained. One disadvantage that cannot easily be recognized in scientific papers is a fact that relatively high levels of polymer chemistry are needed in the polymeric micelle studies. As illustrated in **Figure 1**, an AB type of block copolymer is one of the most favorable structures for the formation of polymeric micelles possessing well-defined shape, size and diameter distribution. The architecture of the AB block copolymer is very simple (two polymer blocks are connected in a tandem manner), but in general its synthesis is more difficult than that of random polymers, where different units are aligned on a polymer chain in a random manner. (As a complete explanation of the difficulties and the limitations of block copolymer syntheses would require more space than is available for this paper, please see other references or textbooks on polymer chemistry.) As a result of the synthesis-related difficulty, choices of monomer units and their combinations are considerably limited. In some cases where laboratories can synthesize a desired block copolymer, it is difficult to synthesize the block copolymer on a large industrial scale in a highly reproducible manner. (In contrast to this general situation, a limited number of block copolymers for drug carriers are produced on an industrial scale and are commercially available. The typical example is Pluronics, poly(ethylene oxide)-*b*-poly(propylene oxide)-*b*-poly(ethylene oxide) block copolymers.) In addition to the above-mentioned problem, some studies have reported cases in which substantial optimization relative to the chemical structures and the chain lengths of the block copolymers was essential for successful drug targeting [61,62,63,68]. This finding indicates that high levels of polymer chemistry are critical for the syntheses of the controlled chemical structures and chain lengths.

Table 1. Advantages of polymeric micelle drug carrier systems.

1. Small diameter with narrow distribution (10 – 100 nm)
2. High structural stability
3. High water solubility
4. Low toxicity
5. Separated functionality

The other disadvantage of the polymeric micelle systems is the immature technology for drug incorporation in a physical manner. Yokoyama *et al.* reported that physical incorporation efficiencies were dependent on drug incorporation methods [61]. At present, there seems to be no universal incorporation method applicable to any polymer. Therefore, researchers must find an appropriate incorporation method for each drug through trial and error. Furthermore, in some methods the drug incorporation may be difficult on a large industrial scale, but easy and efficient on a small laboratory scale. The scale problem is more serious than the polymer synthesis matter because physical factors (e.g., diffusion, solvent exchange rate) are influenced very strongly by the scales in the drug incorporation processes, such as solvent exchange through dialysis membrane. Therefore, more scientific and engineering studies are necessary for significant development on the incorporation technology.

Also, it is worthwhile mentioning that the control of micelle dissociation and drug release rate is essential for drug targeting, and that this control of these matters is sometimes technically difficult to optimize for the targeting, although this is not a disadvantage of the polymeric micelle systems.

3. Reports concerning toxicities of the polymeric micelle systems and biological activities of carrier block copolymers

This section introduces reports concerning toxicities of the polymeric micelle systems and biological (pharmacological) activities of carrier block copolymers.

3.1 Kabanov's report

Kabanov and his colleagues used a poly(ethylene oxide)-*b*-poly (propylene oxide)-*b*-poly(ethylene oxide) block copolymer (ABA type, commercial name Pluronic®) for their polymeric micelle drug delivery system [8,9,19,69-71]. In this system, the block copolymer worked as a biologically active agent rather than as a carrier to specific sites. They reported that Pluronic polymers specifically inhibited the ATP production in mitochondria. P-glycoprotein, which plays an important role in multi-drug resistant cancer cells, shows the efflux action of anticancer drug in an ATP-dependent manner. Consequently, the drug efflux action is inhibited by a Pluronic polymer through the inhibition of ATP production. By using this block copolymer, they reported successful circumvention of the multi-drug resistance both *in vitro* and *in vivo*. This is an innovative application of a synthetic polymer to cancer chemotherapy.

This activity of the block copolymer is very interesting and rather unexpected, as Pluronic polymers lack the functional groups (e.g., charged groups such as carboxyl and amine groups and bulky hydrophobic groups such as long acyl groups) that are expected to have strong interactions with proteins. Kabanov *et al.* showed that occurrences of this activity featured Pluronic polymers possessing an appropriate hydrophilic/hydrophobic balance and appropriate chain lengths. Furthermore, research has not shown any other synthetic polymer that shows P-glycoprotein inhibitory activity. Consequently, it seems that this very interesting and useful activity for cancer chemotherapy is obtained with a very limited number of polymers. However, researchers must pay attention to biological activities of carrier polymers because the mitochondrion and P-glycoprotein inhibitory activities run the risk of raising the levels of toxic side effects in the normal organs and tissues irrespective of the success that Kabanov *et al.*'s animal cancer model had in obtaining enhanced *in vivo* anticancer activity. The presence of this activity can be found relatively easily in the *in vitro* cytotoxic assays (e.g., through determination of drug influx and efflux amounts into/from cells in the presence/absence of polymers). If researchers observe that an anticancer drug's *in vitro* activity increases in strength owing to the drug's combinatory use with a polymer, an advisable subsequent step is to examine the P-glycoprotein in greater detail.

3.2 Yokoyama's report

Yokoyama *et al.* have reported several polymeric micelle anticancer drug targeting systems since the late 1980s, and most of this research team's reports involved the basic study stage. Therefore, the main focus of these reports was on enhanced anticancer activity, improved biodistribution and pharmacokinetic behavior, and related physicochemical characterizations of the carrier systems, whereas descriptions of the carrier systems' toxicities and of the carrier polymers' biological activities were relatively limited. In this limited situation, the research team reported on three important observations, which merit an explanation and a discussion here.

In a series of papers, Yokoyama and co-workers reported enhanced *in vivo* anticancer activity and tumor-specific drug delivery of an anticancer drug, doxorubicin, with drug incorporation into a polymeric micelle carrier [72-78]. These reports focus mainly on *in vivo* anticancer activity and on tumor targeting; however, the reports treat some important information about toxicity. At this point, some details should be noted concerning the chemical structure of the research team's doxorubicin–micelle system because the polymer structure is correlated with toxicity concerns. **Figure 3B** shows the block copolymer structure. Doxorubicin (DOX) was chemically conjugated to aspartic acid residues of the poly(ethylene

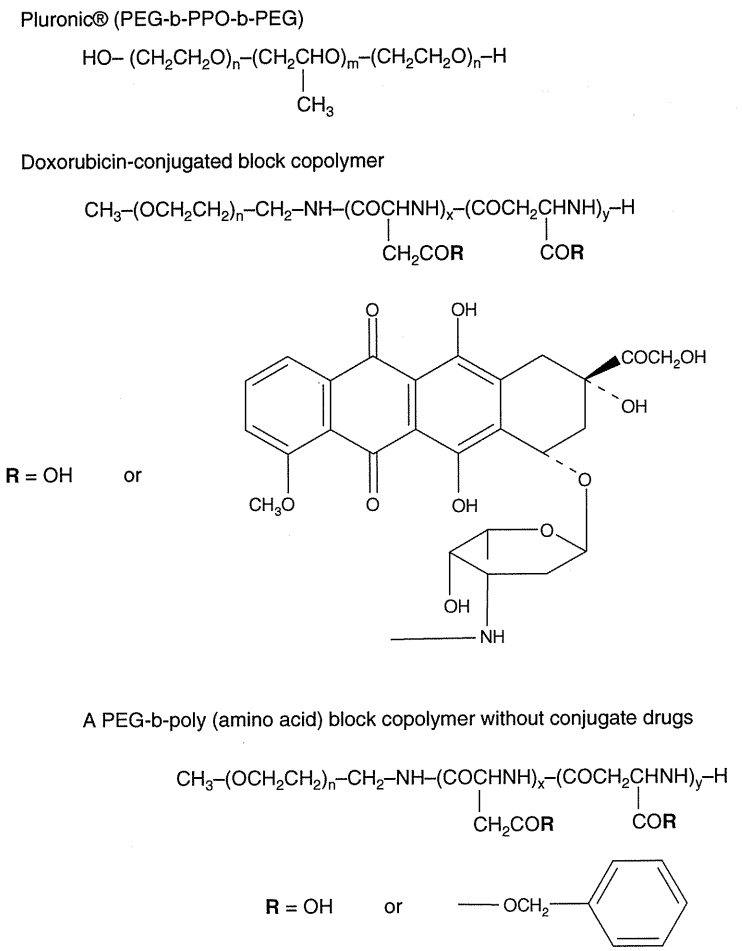

Figure 3. Chemical structures of block copolymer used as polymeric micelle carriers.

glycol)-*b*-poly(aspartic acid) block copolymer through amide bond formation. The poly(ethylene glycol) polymer block was hydrophilic, whereas the DOX-substituted poly(aspartic acid) chain was hydrophobic. Therefore, the obtained drug–block copolymer conjugate (PEG–P(Asp(DOX))) formed micellar structures owing to its amphiphilic character. Doxorubicin was further incorporated into the inner core by means of physical entrapment using hydrophobic and π–π interactions with the chemically conjugated DOX molecules. As a result, polymeric micelles containing both the chemically conjugated and the physically entrapped DOX in the inner core were obtained with the PEG outer shell. Yokoyama *et al.* reported that the carrier polymer PEG–P(Asp(DOX)) did not show any *in vitro* or *in vivo* activities, and that the cytotoxic and anticancer activities were obtained with physically entrapped DOX [78]. The inactivity of the chemically conjugated DOX resulted from the fact that DOX molecules were directly conjugated, without any spacer, to the aspartic acid residues of the block copolymer through amide bonds. These 'direct' amide bonds were considered to be too stable for its cleavage, which provides pharmacologically active free DOX. Therefore, for the purpose of anticancer activity assays, the block polymer can be considered only a carrier, not a biologically active species. On the other hand, attention must be paid to the *in vivo* toxicities attributable to the polymers conjugating drug molecules, particularly in chronic toxic side effects that are not examined in simple toxicity assays done in the basic studies focusing on anticancer activity evaluations.

In another report [76], Yokoyama *et al.* analyzed pharmacokinetic behaviors of the DOX polymeric micelle system as well as enhanced *in vivo* antitumor activity. In this analysis, they showed tumor-selective delivery; the accumulated DOX physically entrapped in the micelle was an amount approximately ninefold larger than the amount of free DOX, whereas the accumulation of the micellar DOX (physically entrapped) in normal organs and tissues was smaller than or the same as the accumulation of free DOX. This report identifies an interesting pharmacokinetic behavior of the micellar DOX. Within 1 h of intravenous (i.v.) injection, accumulated amounts of the micellar DOX in liver were smaller than those

of free DOX. This inequality indicates that the targeting strategy based on the EPR effect was effective in this targeting system even for liver that is known to possess pores large enough for micelles' extravasation in the liver's vasculature. By contrast, 4 h and later, after i.v. injection, this situation was reversed. The micellar DOX showed larger accumulation amounts in liver than free DOX. This inequality resulted from rapid clearance of free DOX in liver through the liver's metabolic activity for drugs, whereas the micellar DOX concentration in liver did not undergo a significant drop, probably because the physically entrapped DOX in the micelle core was largely protected from the metabolic activity. Consequently, the concentration of the micellar DOX was several-fold larger than that of the free DOX. This fact implies that the nonspecific distribution (e.g., at liver) may be an important concern even for successful targeting systems. In another report [74], Yokoyama *et al.* examined toxic side effects of the DOX polymeric micelle system in a composition similar to that of the former one. For liver toxicity, this research team examined pathological observations as well as alanine aminotransferase (ALT)- and aspartate transaminase (AST)-level measurements in blood on day 10 after the first drug injection. The conclusion of this toxicity evaluation was that the toxic side effects of the DOX polymeric micelle were shown in the same pattern as that of free DOX. This means that the liver toxicity was not especially higher than the other side effects for the DOX polymeric micelle. However, considerable attention must be paid to chronic liver toxicity that could not be evaluated in that report up to day 10.

In this DOX-containing polymeric micelle system, the liver toxicity does not seem to be a serious problem because neither unusual nor unexpected toxic side effects were observed in its Phase I clinical trial [14,79,80]. The appearance of the side effects showed the same pattern as that of free DOX in this human clinical trial, where patients underwent longer observations (e.g., several weeks to several months) than was the case in the report's basic study stage.

As explained above, chronic liver toxicity was not serious for the DOX-containing polymeric micelle system. Most scientists in this field posit that the considerable slowness of the metabolism of the incorporated drug compared with the free drug is a universal phenomenon of polymeric micelle drug carrier systems owing to the isolated drug-containing inner core from the outer aqueous environment, where a liver's metabolic enzymes work. Therefore, chronic liver toxicity merits careful examination not only for the polymeric micelle systems, but also for all nano-sized drug carrier systems such as PEG-coated liposomes.

The second important study is Yokoyama and co-worker's report concerning toxicity evaluation of a polymeric micelle carrier lacking drug incorporation [81]. A micelle-forming block copolymer whose chemical structure is shown in **Figure 3C** had been used as a carrier for an anticancer compound camptothecin [61,62,68,82-85] and synthetic retinoids [86-90] such as all-*trans* retinoic acid. The research team analyzed toxicities by conducting pathological examinations that used rats. No pathological abnormality was found for a considerably high dose (200 mg/kg × 5). The team, however, observed significant activation of the mononuclear phagocytic system (MPS) in several organs such as the spleen and liver. Selective accumulation of the polymeric micelle at the MPS was confirmed in an immunohistochemical analysis. These results were obtained with a drug-free polymeric micelle. By contrast, the team concluded that the MPS suffered considerable damage from incorporated cytotoxic drugs in anticancer drug-carrying polymeric micelles. That is why the MPS activation phenomenon seems less important than the other side effects for cytotoxic anticancer drug-carrying systems. However, this MPS-related phenomenon may be important if the polymeric micelle carrier systems are applied to delivering drugs that are much less toxic than typical anticancer drugs.

The third relevant study for biological activity of polymeric micelle carriers is one reported recently concerning the accelerated blood clearance (ABC) phenomenon [91]. ABC is a phenomenon where clearance rates of carrier systems from the bloodstream are raised substantially at repeated injections [92-95]. This ABC phenomenon has been well studied with PEG-coated liposomes that have long-circulating characters at the first injection. A PEG-coated liposome is injected (the first dose) intravenously at an appropriate dose, and then the same liposome is injected (the second dose) according to an appropriate interval (5 – 7 days). This phenomenon occurs owing to immunological activity induced at the first dose, and the change in the clearance is considerable; for example, a case could arise where 90% of the injected dose is circulating 2 h after the first injection and where < 5% of the injected dose is circulating in blood 2 h after the second injection. As polymeric micelles with PEG outer shells have the same profile as that of the PEG-coated liposomes in terms of the PEG outer layer, it is of great interest to know whether these polymeric micelles induce the ABC phenomenon or not. Yokoyama and Oku *et al.* reported the first observation of polymeric micelles' display of the ABC phenomenon [91]. These researchers injected three kinds of polymeric micelle at the first dose, followed by an injection of a PEG-coated liposome at the second injection. The ABC phenomenon was observed only for one polymeric micelle, it was not observed for the other two polymeric micelles. This indicates that some polymeric micelles can induce the ABC phenomenon. At present, the essential properties of the micelle for ABC induction are unknown and will be elucidated in future studies.

The ABC phenomenon is related to the above-mentioned uptake of carrier systems at the MPS and activation of the MPS in the following two aspects.

(1) The existing research suggests that ABC induction develops at the liver [92,93] or the spleen [94,95], both of which are typical organs relative to the MPS. Therefore, the control of uptake extent at the MPS can be a key factor in ABC induction.

(2) It was reported that the ABC phenomenon ceased when the PEG-coated liposomes contained a cytotoxic drug at animal experimental and human clinical levels. Therefore, the importance of the ABC phenomenon becomes greater when drug carriers are applied to pertinent drugs that are much less cytotoxic than typical anticancer drugs. The application to the less cytotoxic drug is a new and important direction of DDS.

3.3 Clinical trials

There are six widely known clinical trials regarding polymeric micelle drug carrier systems. Table 2 summarizes these trials. The objectives for the use of the polymeric micelles are different among these six examples: tumor targeting is the objective for four examples (a – d) [96,97], solubilization of water-insoluble drugs is the objective for one other example (e), and circumvention of multi-drug resistance (MDR) is the objective for the remaining example (f).

Kabanov *et al.* designed and formulated SP-1049C for MDR circumvention on the basis of this research team's basic studies ([8,9,19,69-71]; see Section 3.1). In this system, an anticancer drug, doxorubicin, was physically encapsulated in the hydrophobic inner core of the Pluronic micelles. After being intravenously injected, however, doxorubicin is very rapidly released from the micelle. Owing to this rapid release, concentrations in blood and pharmacokinetic behaviors of the SP-1049C's doxorubicin were almost the same 5 min post-injection as those of the free doxorubicin injection case. I consider that this rapid release stemmed from two factors: the Pluronic micelle inner cores were of relatively low hydrophobicity and of the fluidic state. In the SP-1049C system, the anticancer drug and the biologically active block copolymer are delivered separately to solid tumor cells, and then the MDR circumvention effect will take place there.

Genexol-PM incorporates paclitaxel in a micelle formed from the poly(ethylene glycol)-*b*-poly(DL-lactide) block copolymer [20-23,42]. This micelle possesses a glassy inner core composed of the poly(DL-lactide) block, but drug release in blood occurred very rapidly after intravenous injection, in almost the same manner as that of SP-1049C. Consequently, the pharmacokinetic behavior of the incorporated drug paclitaxel is almost the same as that of the conventional formulation (Taxol). However, Genexol-PM can be superior to Taxol in terms of safe solubilization of water-insoluble drugs. The conventional formulation, Taxol, contains a large amount of surfactant called Cremophor EL. This surfactant has the function of making paclitaxel soluble in water, but accompanying this function is a set of substantial toxic side effects resulting from this surfactant's toxicities. The poly(ethylene glycol)-*b*-poly(DL-lactide) block copolymer is practically a non-toxic substance and successfully plays a role in the solubilization. Therefore, the solubilization is of great value to cancer chemotherapy. As a large incorporation capacity for hydrophobic drugs is a strong advantage of polymeric micelle carriers, more and more drugs may be applied to the polymeric micelle systems for the drug solubilization purposes.

In clinical trials, Japanese, US and British teams examined the remaining four polymeric micelles in relation to solid-tumor targeting. Chemical structures of the inner-core-forming polymer blocks vary depending on the incorporated drug, whereas the poly(ethylene glycol) chain is used for the outer shell in all cases. Tumor targeting is the primary objective of these carrier systems. However, these systems possess another objective: the system has a function to solubilize a water-insoluble drug in the same manner as does Genexol-PM. Matsumura and co-workers reported that NK-105 incorporating paclitaxel showed highly tumor-selective delivery in murine tumor models [14,15,18,40,41]. In clinical stages, NK-105 can have two solubilization-related advantages over the conventional paclitaxel formulation, Taxol. The first advantage attributable to NK-105 is its relatively low toxic side effects, which reflect the fact that the block copolymer is much less toxic than Cremophor EL used in Taxol. The second advantage attributable to NK-105 is that it does not need the premedication that Taxol requires for reducing its own side effects.

We must wait for the final results of Phase II and III trials to answer the question 'Are the polymeric micelle systems valuable in cancer chemotherapy?' While waiting for the answer, we can take comfort in the fact that Phase I clinical studies have already yielded important information concerning toxic side effects. Even for targeted drugs, serious side effects arise because doses are escalated until dose-limiting toxicities become observable. The important obtained information revealed the toxicity profiles of the polymeric micelle drugs, which turned out to be the same as those of the corresponding free drugs. Most of the toxic side effects of the polymeric micelle drugs appear to result from the carriers' release of the drug in the bloodstream. The absence of uncommon and unexpected types of toxicity is a very meaningful fact that can contribute to the safety of clinical use. Not enough clinical results have been obtained to draw a general conclusion that synthetic block copolymers can be safely used in clinical stages, at least for cancer chemotherapy. However, basic study researchers and clinicians must develop their studies while keeping in mind this potential clinical advantage of a drug carrier.

4. Conclusion

In drug targeting with polymeric micelle drug carrier systems, research has reported less information concerning the toxicities of this carrier system than concerning either the systems' therapeutic activities or the systems' pharmacokinetic behaviors. However, even in this limited information, valuable characteristics of the polymeric micelle (no incidence of unexpected or serious toxicities such as hand-and-foot syndrome and toxicities of Cremophor EL) can be known, and these characteristics must be used for further development of

Table 2. Polymeric micelle anticancer drug carrier systems in clinical trials.

Code	Trade name	Primary objective	Incorporated drug	Progress	Company	Refs
a	NK-911	Targeting	Doxorubicin	Phase II	Nippon Kayaku Co., Japan	[14,15,17]
b	NK-105	Targeting	Paclitaxel	Phase II	Nippon Kayaku Co., Japan	[14,15,18,40,41]
c	NC-6004	Targeting	Cisplatin	Phase I	Nanocarrier Co., Japan	[14,15,96,97]
d	NK-012	Targeting	SN-38	Phase I	Nippon Kayaku Co., Japan	[14,15,44-49]
e	Genexol-PM	Solublilization	Paclitaxel	Phase II	Samyang Corp., Korea	[20-23,42]
f	SP-1049C	Anti-MDR effect	Doxorubicin	Phase II	Supratek Pharma, Inc., Canada	[8,9,19,68-70]

the carrier system. Further, some micelle-forming block copolymers show unique pharmacological activities, which perhaps can serve as a basis for new and innovative chemotherapeutic strategies.

5. Expert opinion

In this section, important points are summarized.

(1) Purpose of carrier use. Most pertinent studies state that the use of polymeric micelle carriers serves a drug-targeting purpose, although a considerable proportion of clinical trials are aimed at the other purposes describe below. The drug targeting with polymeric micelle carriers is of high potential owing to the carriers' several physicochemical advantages, such as very small diameters. Two more purposes, solubilization of water-insoluble drugs and circumvention of multi-drug resistance, are unique and valuable regardless of whether or not they accompany the targeting purpose.

(2) Pharmacological activities of block copolymers. Researchers must check whether or not micelle-forming block copolymers alone show any pharmacological activity in the basic study stage, although this type of activity-positive case is not so common according to previous reports. This activity can be evaluated by means of *in vitro* cell culture examinations because drug incorporation generally lowers *in vitro* drug activities for the activity-negative cases. If enhanced or considerably high activities are found, researchers are encouraged to conduct not only further *in vitro* studies to elucidate mechanisms of the activity, but also *in vivo* examinations.

(3) *In vivo* toxicities of the polymeric micelle drug carrier systems. *In vivo* toxicities and their profiles of the polymeric micelle drug carrier systems must be carefully observed in the basic study stage even if some toxicities are so mild that they do not qualify as dose-limiting toxicities. The information concerning the toxicities is valuable in developments for the clinical trial stage even though the information is such that the toxicity profile is the same as the profile of the corresponding free drug.

(4) Some features of the polymeric micelle drug carrier systems in clinical trials. At present, clinical results of the polymeric micelle drug carrier systems are obtained in a very limited manner as compared with liposomal drug carrier systems. Even in this situation, the fact that neither unexpected nor very different profiles of toxicities have been observed is a very valuable one. This is a potentially great advantage of the polymeric micelle system, but many more clinical and basic examinations are required for satisfactory proof of this advantage.

Declaration of interest

M Yokoyama may receive financial benefits from patents NK-911, NK-105 and NC-6004 (summarized in Table 2) when these pharmaceutics go to market after their approval.