—Review—

ナノ医薬品開発に関する動向

加藤くみ子

Development Trend of Nanomedicines

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Nanotechnology has had a great impact on science, technology, and society since 2000, and its applications in medicine are also progressing in the diagnosis, treatment, and prevention of disease. In this review, international trends in nanomedicine regulation are introduced, including the definition of nanomedicines and the evaluation of liposomes and iron nanoparticles.

Key words——nanomedicine; definition; liposome; iron nanoparticle; comparability

1. はじめに

ナノテクノロジー（超微細加工技術）という言葉が大きく取り上げられる発端となったのは，おそらく2000年より米国で開始された国家ナノテクノロジー・イニシアティブ（National Nanotechnology Initiative; NNI）プログラムであろう．これは，国家としてナノテクノロジーの責任ある開発を支援することを目的としており，このプログラムの中で“ナノ”とは「ユニークな現象が新規の応用を可能にする，約1から100 nmのマテリアル」と定義されている.[1] わが国においても，翌年2001年から開始された第2期科学技術基本計画の中で，ナノテクノロジーの医療への応用を推進することが示され，これを受けて厚生労働省においては5ヵ年計画により厚生労働科学研究費補助金・萌芽的先端医療技術推進研究（ナノメディシン分野）（2002–2006）が開始された．このプロジェクトは，ナノテクノロジーを活用した医療技術等の研究開発（ナノメディシン）で，患者にとってより安全・安心な医療技術の実現を図るため，ナノテクノロジーの医学への応用による非侵襲・低侵襲を目指した医療機器等の研究開発を推進するものである．主なプロジェクトとしては，1）微細画像技術の研究開発と，医療分野への応用，2）微小医療機器及びその操作技術への応用，3）薬物送達システム（drug delivery system; DDS）への応用の3項目が取り上げられた．以降，2007–2009年に医療機器開発推進研究事業（ナノメディシン研究），2010年からは医療機器開発推進研究事業（活動領域拡張医療機器開発研究事業と統合）と，現在に至るまで研究支援が継続されている．これらの政策により，わが国におけるナノメディシンに係わる研究が急速に促進されることとなった．このように「ナノメディシン」は，ナノテクノロジーを応用した医療行為や技術，概念そのものを示す言葉であり，ナノテクノロジーを応用した医薬品「ナノ医薬品」を包含する．近年，DDS製剤の主要要素技術である「薬剤放出技術（徐放化や外部刺激応答システムなど）」，「薬剤標的化技術（能動的，受動的標的化技術など）」，「薬物吸収改善技術（プロドラッグ化，新規投与経路など）」のいずれにおいてもナノテクノロジーが利用されつつあり，ナノテクノロジーを応用した医薬品は「ナノDDS製剤」とも呼称されている．本稿では，ナノ医薬品開発に関する国際的な動向について紹介，考察したい．

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2. ナノ医薬品とは

ナノ医薬品の範疇は，各地域の規制当局，さらに学術的な視点により異なると考えられる．以下に欧米規制当局から発出されているナノテクノロジーあるいはナノテクノロジー応用製品に関する定義，作業定義，考慮すべき点（Points to consider）を紹介したい（Table 1）．

2-1. 欧州医薬品庁（European Medicines Agency; EMA） EMAでは，医薬品の品質・安全性・有効性の評価において中心的な役割を担っているヒト用医薬品委員会 Committee for Human Medicinal Products（CHMP）において，2006年に Reflection paper on nanotechnology-based medicinal products for human use[2] が発出されている．これは，昨今のナノテクノロジーを応用した医薬品の開発状況に応じ，EMAの考えを示したものである．「ナノスケールのサイズにすること自体はかならずしも新規性を有するものではないが，ナノテクノロジーにより革新的な医薬品が創出されることが期待されている．これらの製品は医薬品と医療機器との規制上の境界にまたがる可能性もあり，規制上の分類や評価区分に新たな課題をもたらし得る．このようなナノメディシンの品質，有効性，安全性リスクアセスメント評価には専門知識が結集される必要があり，これらの知識の蓄積により新たなガイドラインを作成し，あるいは既存のガイドラインを改定する必要性について精査されるべきであろう．ナノメディシンを開発している申請者は開発の初期から EMA に相談することが推奨される」との内容が記載されている．さらに本文書の中でナノテクノロジーの定義は，以下のように記載されている．

“ナノテクノロジーとは，物質の形やサイズをナノスケールで制御することにより構造，機器，あるいはシステムを作り出すこと，あるいは適用することである”．ここで“ナノスケール”とは，“原子レベルのサイズ 0.2 nm から 100 nm まで”としている．また“ナノメディシン”とは，ナノスケールの物質が有する新規なあるいはさらに価値が向上した物理的，化学的，生物学的特性を十分に引き出し，診断や疾病の治療・予防のためにナノテクノロジーを応用することであるとしている．本文書の中で，ナノメディシンの例としてリポソーム製剤（Caelyx, Myocet），ポリマー結合タンパク質製剤（Peg-intron, Somavert），高分子からなる医薬品（Copaxone），ナノ結晶製剤（Rapamune, Emend）が挙げられている．しかし，ナノメーターサイズではあるが，既に確立された手法により生産された可溶性高分子（組換えペプチドや核酸等）はかならずしも含めないとしている．

Table 1. Control Subject and Condition of Nanotechnology by Regulatory Agencies

Regulatory agency	Year	Control subject and conditions
EMA	2006	Control subject: Production and application of structures, devices and systems Condition: ① “Nanometer scale” ranges from the atomic level at around 0.2 nm up to around 100 nm. ② “Nanomedicine” is defined as the application of nanotechnology in view of making a medical diagnosis or treating or preventing diseases. It exploits the improved and often novel physical, chemical and biological properties of materials at nanometre scale.
Health Canada	2010	Control subject: Manufactured product, material, substance, ingredient, device, system, or structure Condition: ① It is at or within the nanoscale (1 to 100 nm, inclusive) in at least one spatial dimension, or ② It is smaller or larger than the nanoscale in all spatial dimensions and exhibits one or more nanoscale phenomena.
FDA	2011	Control subject: Engineered material or end product Condition: ① Whether an engineered material or end product has at least one dimension in the nanoscale range (approximately 1 nm to 100 nm), or ② Whether an engineered material or end product exhibits properties or phenomena, including physical or chemical properties or biological effects, that are attributable to its dimension(s), even if these dimensions fall outside the nanoscale range, up to 1 μm.

2-2. カナダ厚生省（Health Canada） カナダ厚生省においては，ナノ医薬品に関する情報収集を目的として，暫定的にナノマテリアルに対する“作業定義”を定め，医薬品の治験申請及び新薬申請時にナノマテリアルの含有に関する記載を求めている.[3] この作業定義では，以下の条件に該当するいかなる製造された製品，物質，材料，添加剤，機器，システムあるいは構造をもナノマテリアルとみなしている．つまり，

- それらが，少なくとも一次元においてナノスケール以内であること.

あるいは

- すべての次元が，ナノスケールより小さいかあるいは大きく，かつ1つあるいはそれ以上のナノスケールの現象を有すること.

また，この作業定義の目的で

- “ナノスケール”とは，“1 nm以上100 nm以下”を意味し，
- “ナノスケールの現象”とは，そのサイズに起因する工業製品，物質，材料，添加剤，機器，システムあるいは構造の特性であり，個々の原子・分子・バルク物質が有する化学的，物理的特性とは区別し得る特性である.

さらに

- “製造された”とは，ナノスケールでの工学過程，物質やプロセスの制御を意味する.

とされている.

2-3. 米国食品医薬品局（Food and Drug Administration; FDA） FDAにおいては，2006年にナノテクノロジー・タスクフォースを組織した．このタスクフォースは，ナノスケール・マテリアルを使った革新的で安全で有効なFDA規制対象製品の継続した開発を可能にする規制アプローチを決定する責任を負っている.[4] ここで，FDAが規制する製品には医薬品，生物学的製剤，医療機器並びに食品及び着色剤といった市販前承認を必要とする製品と，栄養補助食品，化粧品，及び食品原料成分といった市販前非承認製品とがある．2011年にこのナノテクノロジー・タスクフォースは“Considering Whether an FDA-Regulated Product Involves the Application of Nanotechnology（FDAが規制権限を持つ製品にナノテクノロジーが応用されているかどうかの検討）”[5] に関するドラフトガイダンスを発出している．これは，FDAが規制する製品がナノマテリアルを含んでいるかどうか，又はナノテクノロジーを応用しているかどうかについてのFDAの現在の考え方を示したものであり，規制目的の定義を定めたものではないとしている．FDA規制対象製品がナノマテリアルを含んでいるかどうか，又はナノテクノロジーを応用しているかどうかについて検討するポイントは以下の通りである.

1. ある工業マテリアル又は最終製品の少なくとも一次元の寸法がナノスケールの範囲（約1 nmから100 nm）かどうか.

又は

2. たとえ寸法がナノスケールの範囲外（ただし最大1 μm）であったとしても，ある工業マテリアル又は最終製品が，その寸法に起因する，物理化学的特性又は生物学的影響を含む特性又は現象を示すかどうか.

これらの検討事項は新しい製品だけでなく，製品又はその成分の寸法，特性，又は効果を変える製法の変更があったときも適用し得る，としている．ここで，工業マテリアル又は最終製品を対象としているのは，偶然含まれる，又はバックグラウンド・レベルのナノマテリアルを含む製品やナノスケールの範囲で自然発生したマテリアルを含む製品から，“意図的”にナノスケールのマテリアルを含む又はナノテクノロジーの応用を含む製品を区別するためである．さらに小さなスケール（ナノスケールを含む）で自然に存在するかもしれない微生物又はタンパク質などの生物又は化学物質をはっきり区別すべきことが述べられている．これらの除外規定は，粒子サイズを意図的に操作し又は制御することにより新たな特性又は現象が生成することは更なる評価に値するとの考えからである．また，“約1 nmから100 nm”というサイズは，消費者製品に関する欧州科学委員会，欧州委員会，上述のカナダ厚生省，国際標準化機構（ISO），経済協力開発機構（OECD）のナノテクノロジーに関するワーキングパーティー，国立癌研究所，米国規格協会等により発表された定義，作業定義，又は説明で使われている．これらの状況を考慮し，FDAが規制権限を持つある製品がナノマテリアルを含んでいるかどうか，又はナノテクノロジーの応用が含まれているかどうかを検討する最初の参照ポイントとして“約1 nmから

100 nm というスケールが適用されるべきとしている．さらに，“その寸法に起因する，物理化学的特性又は生物学的影響を含む特性又は現象”としているのは，ナノスケールのマテリアルが有する特性あるいは現象が，FDA 規制対象製品の安全性，有効性，パフォーマンス，品質，さらに公衆衛生へ影響を及ぼし得るからである．これらナノスケールに起因した特性又は現象のうち医薬品に関連したものとしては，生物学的利用能の増加，用量の減少，又は薬剤の効能の上昇，薬剤の有害性減少などが挙げられている．また，上限として 1 μm を設定した根拠として，1) 寸法に起因する特性を持っているかもしれないが，ナノテクノロジーに関連する可能性が低いマイクロスケールのマテリアルを除外するため，2) 100 nm 以上の寸法を持つが，マイクロスケールのマテリアルとは区別し得るナノテクノロジーに起因した特性又は現象を示すかもしれないマテリアル（例えば凝集体であるアグリゲートやアグロメレート，表面被覆されたもの，官能基が結合されたもの，又は階層的に組み立てられた構造）を含めるためであることを述べている．

日本ではナノテクノロジーあるいはナノ医薬品に関する定義はないため，上記の欧米の文書に照らし合わせ，日本で認可されたナノ医薬品と考えられる主な製品例を表にした（Table 2）．これらに加え，現在日本発のナノメディシンとして注目されている製剤にブロック共重合体ミセル製剤がある．[6]

3. 製品別の規制に関する国際動向

ナノ医薬品に関連した製品別の規制文書は，FDA と EMA から発出されている（Table 3）．

3-1. FDA

① 2002 年 “Guidance for Industry; Liposome Drug Products–Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation, Draft（Table 4）[7]

品質（米国では chemistry, manufacturing and control（CMC）という言葉が用いられる）関連項目では，製剤組成，製品の品質を左右する重要な品質特性パラメータの特定及び測定，製造工程パラメータの特定及びその管理の重要性，有効成分及びリポソームの主原料である脂質の管理，製剤の安定性試験，製法変更時の留意事項が記されている．本ドラフトガイドラインでは，リポソーム製剤のキャリアに相当する脂質成分は添加剤として記載されているが，脂質に関する厳重な管理（特性解析，製造，規格，安定性）を重視している．これは，脂質成分が，リポソームのサイズやリポソームの剛性

Table 2. Examples of Nanomedicines Approved in Japan[a]

Liposomes	AmBisome, Doxil, Visudyne
Polymer-conjugated drugs	SMANCS
Nanocrystal drugs	EMEND
Other nanoparticle drugs	Abraxane, Resovist

[a] Because nanomedicines have not been defined in Japan, products containing materials in the submicron range are listed as “nanomedicines” in this table.

Table 3. Regulatory Documents Relating to Nanomedicines in FDA and EMA

FDA
● Draft Guidance for Industry; Liposome Drug Products Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation (2002)
● Draft Guidance on Doxorubicin Hydrochloride (2010)
EMA
● Reflection paper on non-clinical studies for generic nanoparticle iron medicinal product applications (2010)
● Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product, Draft (2011)

Table 4. Guidance for Industry; Liposome Drug Products–Chemistry, Manufacturing, and Controls; Human Pharmacokinetics and Bioavailability; and Labeling Documentation

CHEMISTRY, MANUFACTURING, AND CONTROLS
A. Description and Composition
B. Physicochemical Properties
C. Description of Manufacturing Process and Process Controls
D. Control of Excipients: Lipid Components
E. Control of Drug Product: Specifications
F. Stability
G. Changes in Manufacturing
HUMAN PHARMACOKINETICS AND BIOAVAILABILITY
A. Bioanalytical Methods
B. *In Vivo* Integrity (Stability) Considerations
C. Protein Binding
D. *In Vitro* Stability
E. Pharmacokinetics and Bioavailability

(rigidity) 等の物理的化学的特性に，さらには薬物の内封量，有効成分の放出性，血中滞留性などの製品としての特性と直接リンクするためである．一方，ヒトでの薬物動態試験とバイオアベイラビリティに関する項目では，1) 生体試料の分析手法，2) *In vivo* での完全性 (integrity) の分析，3) 血中タンパク質の結合，4) *In vitro* 安定性，5) 薬物動態試験とバイオアベイラビリティに関する留意事項が記載されている．*In vivo* での integrity の分析，血清タンパク質の結合の解析により，内封薬物が安定的にリポソーム内に存在し漏出しないか，また血漿タンパク質の結合と細網内皮系（肝臓・脾臓）による取り込みを最低限に抑え得るか解析することにより，血漿中での滞留性に係わる情報を得られるとともに，薬効，及び初期バーストや輸注反応等の有害作用に係わる情報を得られると考えられる．

② 2010 年 "Draft Guidance on Doxorubicin Hydrochloride"[8]

ドキソルビシン塩酸塩含有 PEG 修飾リポソーム製剤の後発品開発における生物学的同等性評価ドラフトガイダンスが発出されている．本ドラフトガイダンスは，参照 PEG 修飾リポソーム製剤と製剤組成，有効成分であるドキソルビシンのリポソームへの内封方法（硫酸アンモニウム勾配法），さらに物理的化学的特性（内封薬物の物理的な存在状態，表面電荷，PEG 層の厚さ，*in vitro* の漏出など）が同等であることを前提とした，非常に限定された製剤についての生物学的同等性評価に関する文書となっている．ドキソルビシンの内封方法については，4 つの主要な手順が示されており，これらすべての手順がうまくいけば，95%以上のドキソルビシンの内封が可能となり，安定したドキソルビシン内封と同時に腫瘍組織でのドキソルビシンの放出が期待されることが報告されている．[9] 生物学的同等性試験として，卵巣がん患者を対象とした臨床試験（遊離のドキソルビシンと内包されたドキソルビシンの AUC と C_{max}），及び *in vitro* 試験として粒子径分布測定が記載されている．物理化学的特性及び *in vivo* におけるリポソーム製剤の重要な特性を理解するとともに，物理的化学的特性に関する分析手法の開発が後発品開発の上で重要であると考えられる．

3-2. 欧州医薬品庁（European Medicines Agency; EMA） EMA から発出されているナノ医薬品関連文書では，非臨床試験について詳細に記述されている点が特徴的である．

① 2010 年 "Reflection paper on non-clinical studies for generic nanoparticle iron medicinal product applications"[10]

EMA からは，MRI 造影剤や鉄欠乏性貧血治療剤として利用されている鉄ナノ粒子製剤の後発品開発における非臨床試験に関するリフレクションペーパー（reflection paper; RP）が発出されている．鉄欠乏性貧血治療剤としての詳細なメカニズムについては明らかになっていないが，数十 nm サイズの鉄多糖類錯体粒子が細網内皮系のマクロファージに取り込まれ，そこで鉄を放出したのち，再び血漿中に出た鉄はトランスフェリンにより骨髄等の作用部位に運ばれ作用すると考えられている．また，マクロファージでの取り込みのほか，肝臓中の内皮細胞等への取り込みも知られている．多くの化学合成医薬品のように循環血流中に吸収された後に薬理作用を示す医薬品の場合は，全身循環血流中に有効成分が同一濃度存在すれば同一の薬理作用を示すと考えられるため，全身循環血流中での有効成分の濃度推移の同等性（バイオアベイラビリティーの同等性）を示すことにより生物学的に同等とされる（つまり生物学的同等性が示されたと言える）．一方，化学合成品を有効成分とする静脈注射製剤では，懸濁剤などの例外もあるが吸収過程を経ないため生物学的同等性試験は必要とされない．しかし多くの静脈注射用ナノ医薬品に関しては，有効成分のキャリアの物理的化学的特性を工夫し循環血中に入った後の有効成分の体内動態（標的部位や主要臓器への分布，代謝，排泄）を制御することを意図した製剤であるため，静脈注射用製剤であっても投与後の体内動態が重要であり，生物学的同等性の比較検証が必要とされることが記されている．さらに，ヒトにおける血中濃度と薬物動態パラメータの比較に基づく生物学的同等性評価のみならず，標的部位や主要臓器における有効成分の測定も安全性，有効性の観点から重要であるが，ヒトにおける試験は通常不可能であるため非臨床試験において標的部位や主要臓器への分布（Biodistribution）を含めた体内動態の比較試験が重要であることの論理的根拠が記されている．特に，鉄ナノ粒子製剤では，少なくとも以下の 3 つのコンパートメント，つまり 1) 血漿，2) 細網内皮

系（reticuloendothelial system; RES）：脾臓・リンパ節・肝臓のクッパー細胞，3）標的組織：薬効に係わる標的組織（例えば骨髄）と毒性に係わる標的組織（例えば，腎臓，肝実質，肺，心臓），に分類し，それぞれにおける鉄ナノ粒子の濃度の比較試験が重要であることが記されている．

② 2011年“Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product”, Draft[11]

さらにEMAからは広く一般の静脈注射リポソーム製剤をスコープとした先発医薬品を参照して開発されたリポソーム製剤に関するRPのドラフト（以下，“リポソームRPドラフト”と略称する）が発出されている．先発医薬品と全く同じではなく，類似の製品を開発する場合は，製品の特殊性に応じてケースバイケースの対応になるが，追加的な非臨床の比較試験（薬物動態試験，組織での有効成分濃度の測定，薬理学的な試験，毒性試験等）が臨床試験による比較検証の前に必要になる可能性が高いことが記されている．

3-3. ナノ医薬品と“comparability（同等性/同質性）”について 上述したEMAのリポソームRPドラフトでは，EMA Guideline on similar biological medicinal products[12]を参照すべき文書として挙げている．キーワードとして“comparability”があり，実際にリポソームRPドラフトでも“comparability”という言葉が多用されている．“Comparability”は日本の規制文書では“同等性/同質性”と訳されている．Comparability（“同等性/同質性”）という言葉は，(1)バイオ医薬品の製法変更前後の製品間の同等性/同質性評価，[13] (2)先行医薬品とのバイオ後続品（欧州ではバイオシミラーBiosimilarと呼称されている）の同等性/同質性評価，において使われる概念である．わが国のバイオ後続品に関する指針[14]の中では，“同等性/同質性”は「先行バイオ医薬品に対して，バイオ後続品の品質特性がまったく同一であるということを意味するのではなく，品質特性において類似性が高く，かつ，品質特性に何らかの差異があったとしても，最終製品の安全性や有効性に有害な影響を及ぼさないと科学的に判断できることを意味する」と説明されている．また，“製品が同等/同質であるか否かを評価する一連の作業”が“Comparability exercise”「同等性/同質性評価作業」である．バイオ後続品評価における「同等性/同質性評価作業」は，

(1) 新薬と同様に，製法確立と品質特性解析．

(2) 品質特性に関する参照先行医薬品（バイオ後続品の場合は先発医薬品ではなく先行医薬品と称する）との比較試験（同等性/同質性評価試験）における類似性の確認．

(3) (2)の結果に応じて，非臨床試験，臨床試験による比較試験（臨床試験は血中濃度の比較に留まらず，有効性，安全性の比較試験をも含む場合が多い）をケースバイケースに行い，後続医薬品が先行医薬品と同等/同質であることを確認．

となる．この同等性・同質性評価作業の基本的な考え方が，先発医薬品を対象として開発されるリポソームRPドラフトに関しても適応されており，通常の後発医薬品における生物学的同等性評価（bioequivalency）という表現は用いられていない．また，“generic”という言葉も用いられておらず，“先発医薬品を参照して開発されたリポソーム製剤”としている．ただし欧州ではヒト医薬品の申請に関する法律（Directive 2001/83/EC）が2003年に改訂され，Similar Biological Medicinal Productというバイオシミラー製品の承認申請のためのカテゴリーが新たに設定されたが，[15] リポソーム製剤では現時点でバイオシミラーのような新たな規制の枠組みは新設されていない．

そこで，EMAリポソームRPドラフトでは，先発医薬品を参照としたリポソーム医薬品開発において，通常の後発医薬品における生物学的同等性評価ではなく，バイオ後続品と同様な同等性/同質性評価のアプローチであることが強調されている理由について考察したい．[16]

一般に化学合成医薬品を主体とした後発医薬品は(1)先発医薬品と有効成分が同一であり，(2)先発医薬品と同一用法，用量で，同等の効能・効果が期待される製剤である．そこで，(1)有効成分の同一性と，(2)製剤の生物学的同等性の視点から，化学合成医薬品，バイオ医薬品との比較を通して，リポソーム製剤の評価について考察したい．

① 有効成分の同一性

通常，「同一性」とは有効成分の化学構造が「完全に一致」している場合に用いられる．しかし，バ

イオ医薬品のほとんどはタンパク質性医薬品であり，有効成分であるタンパク質の同一性を示すには一次構造のみならず，高次構造が同一であることを示す必要がある．高次構造の同一性の確認は現在でも分析手法の限界から技術的に困難なことも多く，対象先発医薬品の原薬を手に入れることも通常困難である．さらに，糖タンパク質などのように翻訳後修飾により分子多様性を示す有効成分の場合，多様な構成物からなるので，同一性を示すことは不可能な製品がほとんどである．したがって，多くのバイオ後続品では「有効成分の同一性」の評価ではなく，「有効成分の類似性」の評価が目標となる．

一方，リポソーム製剤の場合，これまでに承認されているリポソーム製剤の有効成分は低分子合成化合物であり，その同一性を示すことは可能であろう（ただし，例外としてウイルス由来の成分を脂質二重膜の表面に結合させアジュバンドワクチンとして利用されるリポソーム（virosome と呼ばれる）が海外で市販されている）．

② 製剤の生物学的同等性

製剤の生物学的同等性という視点からはどうであろうか．鉄ナノ粒子の RP の項で述べたように「医薬品の作用，効果は作用部位での薬物濃度に依存する」ことから，循環血流中に吸収された後に薬理作用を示す医薬品の場合は，全身循環血流中に有効成分が同一濃度存在すれば同一の薬理作用を示すと考えられる．そのため，通常の化学合成医薬品後発製剤では全身循環血流中での有効成分の濃度推移の同等性（バイオアベイラビリティーの同等性）を示すことにより先発製剤と生物学的に同等とされる．したがって，通常の化学合成医薬品の静脈注射用製剤では吸収過程を経ないため同等性試験は必要とされない．わが国の「後発医薬品の生物学的同等性ガイドライン」[17] では，生物学的同等性を示すことができれば，非臨床・臨床試験による有効性・安全性の確認は必要ないとされている（ただし，血中又は尿中の未変化体又は活性代謝物の定量的測定が困難な医薬品，及びバイオアベイラビリティの測定が治療効果の指標とならない医薬品の場合などは，薬力学的試験での比較，さらに薬力学的試験が困難あるいは適切でないときは臨床試験での比較をすることとなっている）．

一方，バイオ後続品においては，上述の通り有効成分が参照先行医薬品と同一である，という前提で評価できない場合が多く，吸収過程のない静脈内投与注射剤であっても，体内分布，代謝，排泄に違いがある可能性があるため，臨床薬物動態の比較試験は必要とされる．さらに，生体への作用は作用部位での濃度に加え生物活性にも依存するため，血中動態の同等性のみを以って生物学的に同等と判断できない場合が多い．したがって，定量的な評価が可能な有効性の代替マーカーが設定できる場合は，臨床薬力学 pharmacodynamics（PD）試験による比較，あるいは pharmacokinetics（PK）/PD の比較が生物学的同等性を確認する上で重要になる．さらに代替マーカーが設定できない場合は，程度の差はあるが臨床エンドポイントを指標とした臨床有効性試験によらなければならないケースもある．さらに，安全性への影響という観点からは，有効成分のわずかな構造変化が免疫原性の原因となり有害作用を示す可能性があることからも臨床試験が必要となる場合が多い．このように，バイオ後続品の評価では，有効成分の特殊性から血中濃度推移の比較に加え，製品に応じ非臨床試験，臨床試験での比較試験により，総合的に参照先行医薬品との同等性/同質性を評価することとなる．

リポソーム製剤ではどうであろうか．多くの静脈注射リポソーム製剤では，製品により特性は異なるが循環血中に入った後の血中滞留性，放出性，標的性等を制御するように製剤が設計されており，有効成分単独で投与された場合とは体内分布，代謝，排泄ともに大きく変化するため，臨床薬物動態の比較試験は必要とされる．さらに，リポソームを構成する脂質原料及びその組成は，リポソームの形状や粒子径等の高次構造，表面電荷，剛性（rigidity），二重膜の流動性などの物理的化学的特性に影響を与えるが，脂質原料に水素添加大豆ホスファチジルコリンなど天然由来成分を用いている製品や製法が複雑な製品もあり，参照先発医薬品と同じ原料や組成，製造方法に関する情報を得られない場合は，物理的化学的特性や *in vitro* 漏出性等に差を生じる可能性があり，先発医薬品との製剤的な同等性（通常，製剤間の比較の場合は「同一性」ではなく「同等性」を用いる）を示すことは困難となる（したがって類似性を示すことが目標になる）．リポソーム製剤の物理的化学的な特性は，血中滞留性，組織浸透性，

標的組織での有効成分の放出性等の有効性を左右する製品の機能に直接リンクしているとともに，補体活性等の免疫学的な安全性にも関係している[18]ため，参照先発医薬品との物理的化学的特性のわずかな差が有効性や安全性に影響を及ぼす可能性があり，血中動態の同等性のみを以って生物学的に同等と判断できない場合が多いであろうと考えられる．

以上のように，多くの静脈注射リポソーム製剤では製品の薬物動態学的特性（特に，標的組織等への分布 biodistribution）から，また製剤学的に参照先発医薬品との同等性を示すことが困難であるという理由から，ヒトでの血中濃度推移の比較に基づく生物学的同等性評価の前に，動物を用いた血中濃度推移の比較評価，さらに組織分布（標的組織や毒性に係わる組織における有効成分の濃度測定と参照先発医薬品との比較），薬理学的作用や毒性の同等性/同質性評価が必要とされると考えられ，リポソーム RP ドラフトにも強調されている．ただし，製品の特殊性に応じてケースバイケースの対応により非臨床試験の同等性/同質性試験を低減でき，また適切であればある試験を免除できる可能性にも言及されている．さらに臨床試験についても，薬物動態試験のほかに有効性・安全性試験が必要とされるかどうかは，ケースバイケースであり，非臨床試験のモデルや臨床薬物動態に関するデータでどの程度参照先発医薬品との差が検出可能であるか，また製剤処方の特殊性の程度により判断され得るべきであるとしている．

このように，EMA のリポソーム RP ドラフトでは，リポソーム製剤評価においては，通常の低分子合成医薬品の後発医薬品開発で行われている生物学的同等性評価からさらにプラスアルファの評価が必要であり，製品に応じ非臨床試験，臨床試験も行いながら，総合的に同等/同質の有効性・安全性を有することを評価する comparability exercise の考え方が示されている．しかし今後，リポソーム製剤の物理的化学的特性解析技術開発の進展や，体内動態（全身レベルから組織・器官レベル，製品によっては細胞レベル，細胞小器官レベルに至るまで）と品質特性の関係さらには医薬品としての有効性・安全性との関係に関する知識の蓄積により，体内動態に重要な影響を及ぼす製剤の重要品質特性が特定できるようになれば，製品によっては現在の化学合成医薬品後発品と同様な基準による評価が可能になるかもしれない．

4. ナノ医薬品評価に関する今後の課題

わが国においては，現時点でナノ医薬品評価の規制に関連した文書は出されていない．しかし，新規素材をキャリアとした抗がん剤等の標的部位への送達，放出制御を狙った製剤は世界に先駆けて進行中である．したがって，ナノ医薬品の開発，承認申請において配慮すべきポイントを明確にし，さらには評価ガイドライン等としてまとめることが課題となっている．

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Intracellular trafficking mechanism, from intracellular uptake to extracellular efflux, for phospholipid/cholesterol liposomes

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ABSTRACT

Liposomes are widely used as drug delivery vehicles to transfer chemotherapeutic agents, proteins, and nucleic acids into target cells. To improve therapeutic effects and reduce unexpected toxic side-effects, it is necessary to understand the mechanism of liposomal uptake into cells, and the intracellular fate of internalized liposomes. The intracellular fate of synthesized components used in the construction of liposomes remains unclear. In the work presented here, we investigated the trafficking processes from intracellular uptake to extracellular efflux using conventional liposomes constructed with phospholipids (DOPC) and cholesterols (Chol). Following intracellular transport of liposomes *via* endocytosis, DOPC was localized in the endoplasmic reticulum (ER) and Golgi apparatus after escape from the endosome/lysosome, whereas Chol was only localized in the ER. Moreover, proteins involved in the intracellular trafficking of liposomal components were identified. Additionally, we showed that DOPC was partly effluxed *via* ABCG1, while Chol was partly effluxed *via* ABCA1 or ABCB1; suggesting that each liposomal component examined in this study was effluxed through different transporters. Our findings offer valuable information regarding targeted delivery to specific intracellular organelles, and how to possibly avoid unexpected toxic effects following multiple applications of liposome formulations.

1. Introduction

Drug delivery systems using nano-materials are promising technologies for the effective transfer of chemotherapeutic agents, proteins, and nucleic acids. Their use offers improved pharmacokinetic properties, controlled and sustained release of drugs, and lower systemic toxicity. The commercial availability of liposomal doxorubicin (Doxil) and albumin-nanoparticle-based paclitaxel (Abraxane) has focused attention on this innovative and exciting field. This is especially true of liposomes, which are spherical vesicles consisting of a lipid bilayer that can encapsulate various types of drugs into an inner aqueous phase or lipid bilayer [1,2]. These properties of liposomes are expected to enable them to be effective drug carriers. It has been shown that liposomes are able to efficiently accumulate in tumors because of enhanced permeability and retention effects, which are in turn due to an increase in vascular permeability and the poor state of the lymphatic tissue *in vivo* [3]. Therefore, many researchers have studied liposomes as drug carriers, and some liposomal formulations for cancer treatment have been applied clinically. Surface modification of liposomes by water-soluble polymers such as polyethylene glycol, or specific ligands such as antibodies and peptides, have also been studied for tumor-selective drug delivery [4–6].

Following administration into the body, it is known that liposomes with access to target tissues are taken up into cells *via* various types of endocytosis [7,8]. Since nucleic acids, proteins, and peptides are not taken up into cells *via* passive diffusion, the intracellular uptake of liposomes is a key factor for liposome-mediated delivery of these compounds and ensuring they have adequate therapeutic effects. As far as the intracellular transport of liposomes is concerned, many researchers have reported that conventional liposomes are taken up into cells *via* clathrin-mediated endocytosis [8,9]. It has been reported that the physicochemical properties and surface modification of liposomes affect intracellular uptake mechanisms with many findings related to the intracellular uptake of liposomes [10–13].

Nucleus- or mitochondria-selective drug delivery using liposomes has been achieved by modification of specific ligands [14,15]. However, there are fewer reports related to the targeted transport of liposomes into other organelles, such as Golgi apparatus or endoplasmic reticulum (ER). Kheirolomoom and Ferrara reported that endogenous cholesterol (Chol) was taken up into cells *via*

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caveolae-mediated endocytosis [12]. Hao *et al.* reported that endogenous Chol was transported to cell membranes *via* the ER [16]. The elucidation of molecules involved in intracellular transport of endogenous Chol continues [17,18]; however, there are few reports regarding the intracellular transport of synthesized Chol as a liposome component. We have reported the intracellular trafficking of block copolymers from uptake to efflux [19]. Although the intracellular uptake mechanism of phosphatidylcholines has been reported previously [18], there is little literature about their intracellular trafficking mechanisms of liposomes. Thus, details about the intracellular trafficking mechanisms of liposomes and/or their components remain unclear; therefore, the elucidation of the intracellular trafficking process from uptake to efflux of liposomes and/or their components offers valuable information for targeted delivery to various organelles.

It has also been reported that excess accumulation of fatty acids leads to activation of mitochondrial β-oxidation, generating reactive oxygen species involved in tumor growth [20]. Therefore, the elucidation of extracellular efflux mechanisms of liposomal components in cancer cells, especially phospholipids, might contribute to avoiding unexpected tumor growth and toxicity following multiple administrations of liposomal formulations.

In the present study, we selected conventional liposomes composed of DOPC and Chol as a model liposomal formulation, and investigated the intracellular trafficking process from intracellular uptake to extracellular efflux of liposomal components. The intracellular trafficking of DOPC and Chol was evaluated based on the fluorescent intensity of nitrobenzoxadiazole (NBD)-conjugated derivatives according to another report [21]. The intracellular trafficking of liposomal components, from intracellular uptake to extracellular efflux, was evaluated and then factors involved in the intracellular trafficking of liposomal components were determined. The effects of Chol on intracellular trafficking of phospholipids were also investigated.

2. Materials and methods

2.1. Materials and cells

1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC) and Chol were purchased from Sigma–Aldrich (St. Louis, MO, USA). NBD-labeled DOPC and Chol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Dulbecco's modified Eagle's Medium (D-MEM), RPMI-1640, penicillin/streptomycin, and Opti-MEM I were purchased from Life Technologies (Brooklyn, NY, USA), and fetal bovine serum (FBS) was obtained from Nichirei Biosciences (Tokyo, Japan). All other chemicals used in this study were of the highest purity available. HeLa (Health Science Research Resources Bank, Osaka, Japan) and HT-29 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in D-MEM and RPMI-1640, respectively. The media were supplemented with 10% FBS, 100 U/ml penicillin/streptomycin. Cells were grown in a humidified incubator at 37 °C/5% CO_2.

2.2. Preparation of liposomes

Liposomes used in this study were prepared according to the Bangham method [22]. Briefly, DOPC and Chol were mixed in chloroform at a molar ratio of 1:1, and the mixture was dried by evaporation and vacuum desiccation. The resultant lipid film was re-suspended in phosphate-buffered saline (pH 7.4) under mechanical agitation. After hydration for 30 min at room temperature, the dispersion was sonicated for 10 min in a bath-type sonicator (Honda Electronics, Aichi, Japan) and for 3 min in a tip-type sonicator (Sonics, Newtown, CT, USA). Liposomes were sized by repeated extrusion through polycarbonate membrane filters (Avestin, Ottawa, Canada) with a pore size of 100 nm. The particle sizes, polydispersity index (PDI), and ζ-potentials of liposomes were determined by a Zetasizer Nano ZS instrument (Malvern Instrument, UK).

2.3. Evaluation of intracellular trafficking of liposomes

For quantifying intracellular phosphatidylcholines and Chol derived from liposomes, the liposomes containing NBD-labeled DOPC and Chol at a molar ratio of 5.0% were used in this study. Cells (5×10^4) were seeded on six-well plates in medium containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for 48 h at 37 °C/5% CO_2, cells were exposed to 50 μg/mL liposomes in culture medium. After incubation for pre-determined durations, the incubation medium was replaced with Hanks' balanced salt solution (HBSS). Cells were the trypsinized with 0.25% trypsin-ethylenediamine tetraacetic acid (Life Technologies), washed with HBSS three times, and suspended in lysis buffer (1.0% Triton X-100 in HBSS). The cell suspension was then shaken and centrifuged (15,000 × *g*, 4 °C, 10 min), and the fluorescence intensity of the resultant supernatant measured (excitation wavelength, 474 nm; emission wavelength, 533 nm) using a fluorescence spectrophotometer (F-7000; Hitachi High-Technologies, Tokyo, Japan). The fluorescence intensity was normalized with respect to the protein content of cells. The protein concentration was determined using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

2.4. Confocal microscopy

To observe co-localization, endosomes and lysosomes of cells were labeled with AlexaFluor-594-conjugated transferrin (Life Technologies) and LysoTracker Red DND-99 (Life Technologies), respectively, in accordance with the manufacturer's instructions. For labeling of the ER and Golgi apparatus, CellLight (Life Technologies), which is a fluorescent protein-signal peptide fusion for specific labeling of organelles, was used according to the manufacturer's instructions. The confocal microscopy observation was performed according to the previous report [19]. Briefly, cells (1.0×10^5) were plated on 35-mm glass-bottom dishes coated with poly-L-lysine (Matsunami glass, Osaka, Japan) in medium containing 10% FBS and 100 U/mL penicillin/streptomycin. After incubation for 48 h, cells were exposed to 50 μg/mL liposomes in culture medium. At a predetermined time after addition of liposomes containing NBD-labeled DOPC or Chol, cells were washed and kept in HBSS for imaging with a confocal microscope (Carl Zeiss LSM 510; Carl Zeiss Microscopy GmbH, Germany). Pseudocolor luminescent images were captured using LSM Image Browser (Carl Zeiss Microscopy GmbH, Germany).

2.5. Endocytosis inhibition and Golgi destruction

Endocytosis was inhibited using 10 μg/mL chlorpromazine, a clathrin-mediated endocytosis inhibitor; 150 μM genistein or 2.0 mM methyl-β-cyclodextrin, caveolae-mediated endocytosis inhibitors; and 50 μM 5-(N-ethyl-N-isopropyl) amiloride, a macropinocytosis inhibitor [23,24]. Each endocytosis inhibitor was added to culture medium at 30 min before the addition of liposomes. To inhibit ER-to-Golgi transfer, cells were incubated in medium containing 1 μg/mL brefeldin A and 30 μM nocodazole for 30 min before the addition of liposomes [25].

2.6. siRNA transfer

Stealth RNAi oligonucleotides (25 mer) were obtained from Life Technologies. The siRNA sequences used in this study can be seen in Table 1. As a negative control, the Stealth RNAi High GC Negative Control Duplex (Life Technologies) was used [19]. The Stealth RNAi oligonucleotides were transfected into cells using Lipofectamine RNAiMAX (Life Technologies) according to the manufacturer's protocols. At 48 h before the addition of liposomes, each siRNA was added to cells, and incubated for

Table 1
Sequences of siRNAs used in this study.

Target gene	Sense strand	Antisense strand
MLN64	5′-GCUGA AGGAU UAAAC AAUGA CUUCA-3′	5′-UGAAG UCAUU GUUUA AUCCU UCAGC-3′
ORP1	5′-GCACC UCUGA GGAGU UGGAU GAAAU-3′	5′-AUUUC AUCCA ACUCC UCAGA GGUGC-3′
NPC1	5′-CCCUC GUCCU GGAUC GACGA UUAUU-3′	5′-AAUAA UCGUC GAUCC AGGAC GAGGG-3′
CERT	5′-ACGUG AGAAG UUGGC UGAAA UGGAA-3′	5′-UUCCA UUUCA GCCAA CUUCU CACGU-3′
sec31A	5′-CCAGG CCAAU AAGCU GGGUG UCUAA-3′	5′-UUAGA CACCC AGCUU AUUGG CCUGG-3′
ORP2	5′-GAGAG GAGAG GUGAC CACCU GAGAA-3′	5′-UUCUC AGGUG GUCAC CUCUC CUCUC-3′
PITP	5′-GGAUA UUUAC AAACU UCCAU CGCCA-3′	5′-UGGCG AUGGA AGUUU GUAAA UAUCC-3′
ABCA1	5′-UUUAG AUGCU GGACA CUGCC AAGGC-3′	5′-GCCUU GGCAG UGUCC AGCAU CUAAA-3′
ABCB1	5′-UCCCG UAGAA ACCUU ACAUU UAUGG-3′	5′-CCAUA AAUGU AAGGU UUCUA CGGGA-3′
ABCC1	5′-CCGGU CUAUU CCCAU UUCAA CGAGA-3′	5′-UCUCG UUGAA AUGGG AAUAG ACCGG-3′
ABCG1	5′-UCUCG CUGAU GAAAG GGCUC GCUCA-3′	5′-UGAGC GAGCC CUUUC AUCAG CGAGA-3′
snap-25	5′-CAUGG AGAAG GCUGA UUCCA ACAAA-3′	5′-UUUGU UGGAA UCAGC CUUCU CCAUG-3′

6 h. Then, the culture medium was replenished and the cells were incubated for a further 42 h.

2.7. Statistical analyses

Results were presented as the mean ± SD of greater than three experiments. Analysis of variance was used to test the statistical significance of the differences among groups. Two-group comparisons were performed by Student's *t*-test, and multiple comparisons between control and test groups were performed by Dunnett's test.

3. Results

3.1. Physicochemical properties

The physicochemical properties of liposomes containing NBD-labeled DOPC or Chol were evaluated by measuring the particle sizes, PDI, and ζ-potentials. The mean particle sizes and PDI were approximately 110 nm and 0.060, respectively (Table 2). The ζ-potentials of NBD-labeled liposomes were approximately −0.050 mV (Table 2), because of weak anionic properties based on phosphate groups of DOPC. These physicochemical properties of liposomes correspond to those previously reported [26,27].

3.2. Intracellular transport

To investigate the intracellular transport mechanisms of liposomes and their components used in this study, the intracellular amounts of liposomes were quantified in HeLa and HT-29 cells. Liposomes were labeled with NBD-labeled DOPC or Chol, and intracellular uptake of liposomes into cells was studied using confocal microscopy. DOPC and Chol were observed to co-localize with endosomes at 1 h after the addition of each liposome in HeLa cells (Fig. 1A). Following endocytosis inhibitory experiments, the intracellular amounts of liposomes made with DOPC and Chol in HeLa and HT-29 cells were significantly suppressed at 2 h after the addition of each liposome in the presence of chlorpromazine (Fig. 1B). These observations agree with previously published results regarding intracellular transport mechanisms of liposomes with a similar composition [28,29].

3.3. Intracellular localization of DOPC/Chol

We examined the intracellular localization of DOPC and Chol by confocal microscopy. DOPC was co-localized to the ER and Golgi apparatus at 24 h after the addition of liposomes in HeLa cells, while Chol was co-localized to the ER only (Fig. 2). These results suggest that the spherical structure of liposomes might be degraded before liposomes are transported to the ER or Golgi apparatus, and that DOPC and Chol are trafficked separately in the cells. Moreover, these findings also suggest that the intracellular trafficking pathway from the endosome to the ER or Golgi apparatus differs between DOPC and Chol.

Table 2
Particle sizes, PDI, and ζ-potentials of liposomes used in this study. Each value represents the mean ± SD ($n = 3$).

	Particle size (nm)	PDI	Zeta-potential (mV)
DOPC:chol: NBD-labeled DOPC (45:50:5 at a molar ratio)	110 ± 1.6	0.058 ± 0.014	−0.073 ± 0.20
DOPC:chol NBD-labeled chol (50:45:5: at a molar ratio)	111 ± 4.8	0.059 ± 0.009	−0.055 ± 0.19
DOPC:NBD-labeled DOPC (95:5 at a molar ratio)	107 ± 2.3	0.068 ± 0.021	−0.066 ± 0.28

3.4. Trafficking mechanisms from the endosome/lysosome to the cytoplasm

To elucidate the trafficking mechanisms from the endosome/lysosome to the cytoplasm for DOPC or Chol derived from liposomes, the inhibitory effects of metastatic lymph-node gene 64 protein (MLN64) [30], oxysterol-binding protein-related protein 1 (ORP1) [31–33], and Niemann-Pick C1 protein (NPC1) [34] (all lipid transport-related proteins) were evaluated in HeLa and HT-29 cells (Fig. 3A). Under these conditions, when the extracellular efflux of liposomal components was decreased following suppression of specific intracellular transport processes, the intracellular amounts of each liposomal component were increased. As shown in Fig. 3B, the intracellular amounts of DOPC and Chol at 24 h after the addition of liposomes were increased when ORP1 and NPC1 expression were suppressed, respectively. Confocal microscopy revealed that the transport of DOPC and Chol from endosome/lysosome to the ER or Golgi apparatus was suppressed when ORP1 and NPC1 expression were knocked-down, respectively (Fig. 3C). These results suggest that the intracellular trafficking of DOPC and Chol from endosome/lysosome to the ER/Golgi is partly controlled by ORP1 and NPC1, respectively.

3.5. ER-to-Golgi transport in intracellular trafficking of DOPC/Chol

To investigate the involvement of ER-to-Golgi transport in intracellular trafficking of DOPC/Chol, the inhibitory effects of ER-to-Golgi transport-related proteins were examined in HeLa and HT-29 cells. We selected CERT, a known ceramide-transfer protein [35,36], and sec31A, a component of COPII required for vesicle budding from the ER [37,38] (Fig. 3A). Following knockdown experiments, the intracellular amounts of DOPC at 24 h after the addition of liposomes were increased when CERT or sec31A expression were suppressed (Fig. 4A). However, the intracellular amounts of Chol were not affected by CERT or sec31A knockdown (Fig. 4A). Moreover, the intracellular amounts of DOPC were increased in the presence of brefeldin A, an inhibitor of the transport pathway from the ER to the Golgi [25], but was not increased in the presence of nocodazole, an inhibitor of the transport pathway from the Golgi to the ER [25] (Fig. 4B). Transport of DOPC was arrested in the ER by CERT knockdown, but intracellular trafficking of Chol was not affected by CERT knockdown (Fig. 4C). These results may indicate that although DOPC trafficked intracellularly *via* ER-to-Golgi transport, the ER-to-Golgi transport system is not involved in trafficking of Chol.

3.6. Extracellular efflux mechanisms of DOPC/Chol

To investigate the transport of DOPC/Chol to the cell membrane, we closely examined oxysterol-binding protein-related protein 2 (ORP2) [39–41] and phosphatidylinositol transfer protein (PITP) [42–44]. These are both involved in the intracellular transport of lipidic molecules (Fig. 3A). Following knockdown experiments using siRNA against each protein, the intracellular amounts of DOPC at 24 h after the addition of liposomes were increased when PITP expression was suppressed (Fig. 5A). The amounts of Chol were increased when ORP2 expression was suppressed (Fig. 5A). We have already indicated that the ABC transporter is involved in the efflux of doxorubicin-bound block copolymers [19]. Therefore, the involvement of the ATP-binding cassette (ABC) transporter and exocytosis in the extracellular efflux of DOPC/Chol was investigated in HeLa and HT-29 cells. Following knockdown experiments of

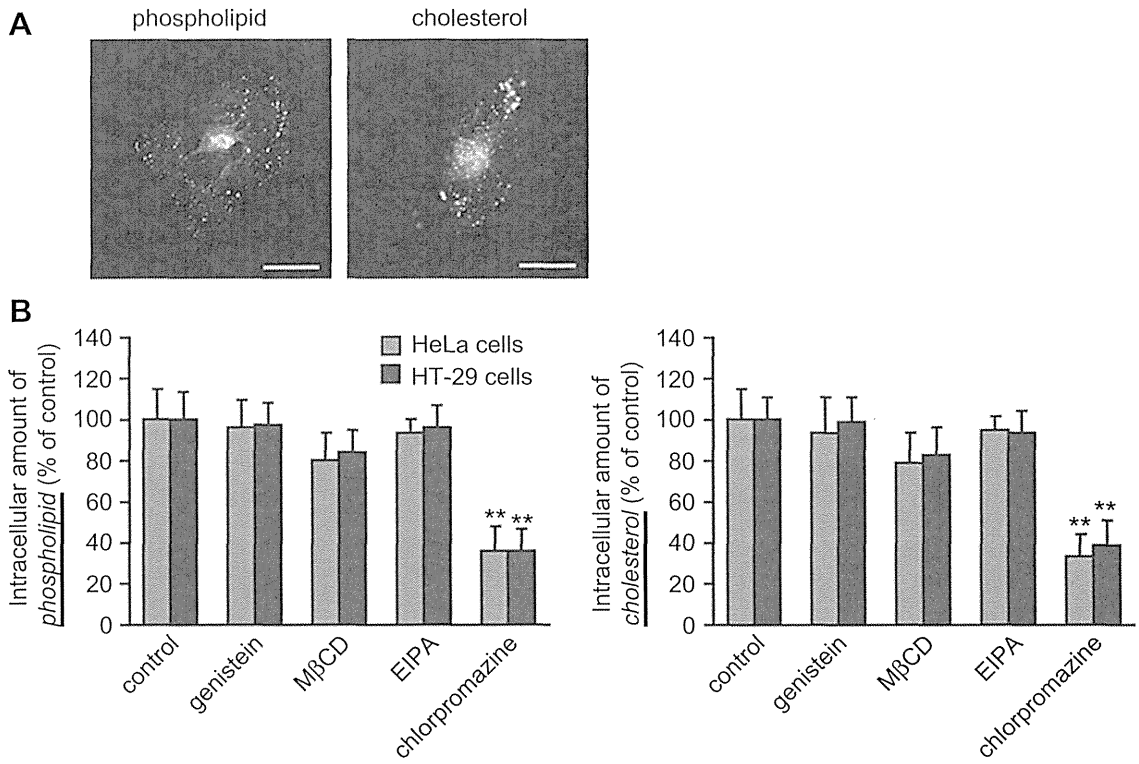

Fig. 1. (A) Confocal images showing intracellular transport of DOPC/Chol at 1 h after the addition of liposomes to HeLa cells. Liposomes were labeled with NBD-labeled DOPC (green) or Chol (green) for separate evaluation of their intracellular localization. The endosomes/lysosomes were labeled with AlexaFluor-594-conjugated transferrin/LysoTracker Red DND-99 (red). Scale bars, 20 μm. (B) The intracellular transport of DOPC/Chol at 2 h after the addition of liposomes in HeLa and HT-29 cells. Each endocytosis inhibitor was added to cells at 30 min before the addition of liposomes. $^{**}P < 0.01$ compared with the corresponding control group. Each value represents the mean + SD ($n = 6$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

various ABC transporters (ABCA1, ABCB1, ABCC1, and ABCG1), the intracellular amounts of DOPC at 24 h after the addition of liposomes were increased when ABCG1 expression was suppressed (Fig. 5B). The intracellular amounts of Chol were increased when the expressions of ABCA1 or ABCB1 were suppressed (Fig. 5B). With respect to exocytosis, following inhibition of snap-25, a known exocytosis-related protein, the intracellular amounts of DOPC and Chol were increased in HeLa cells but not in HT-29 cells (Fig. 5C).

3.7. *Effects of Chol on intracellular trafficking of DOPC*

To investigate the effects of Chol on intracellular transport of DOPC, we used liposomes without Chol. The intracellular amounts of DOPC in HeLa cells were significantly suppressed in the presence of chlorpromazine, and this was independent of Chol (Fig. 6A). Intracellular trafficking processes involving DOPC in HeLa cells were affected by Chol. Following suppression of proteins involved

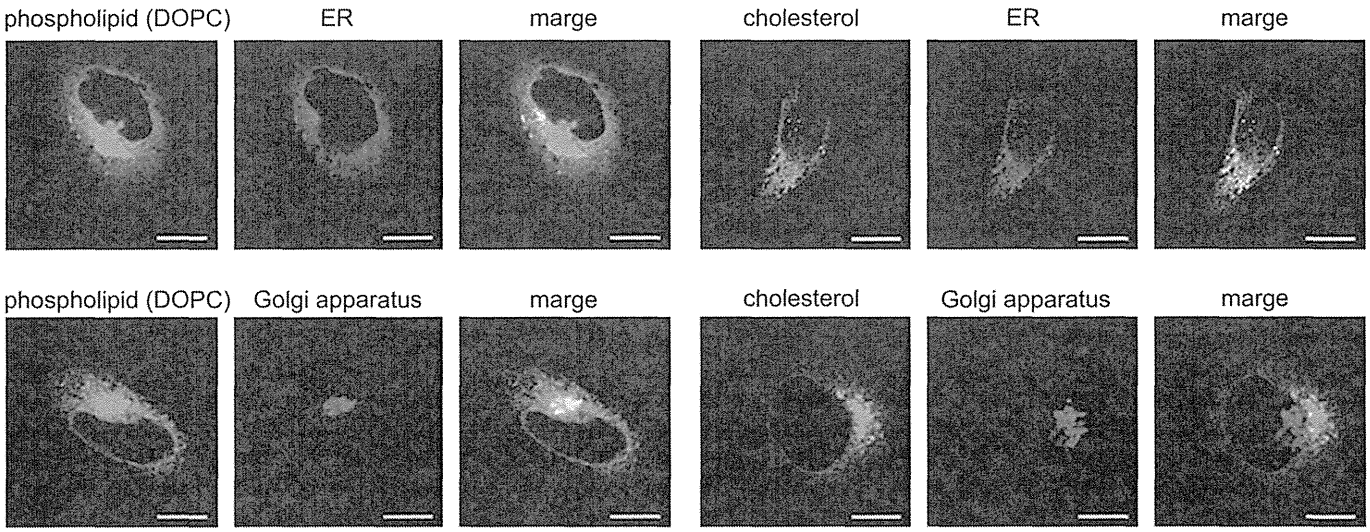

Fig. 2. Confocal images showing the intracellular localization of DOPC/Chol at 24 h after the addition of liposomes in HeLa cells. Liposomes were labeled with NBD-labeled DOPC (green) or Chol (green) for separate evaluation of the intracellular localization of each component. ER and Golgi apparatus were labeled with CellLight-RFP (red). Scale bars, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

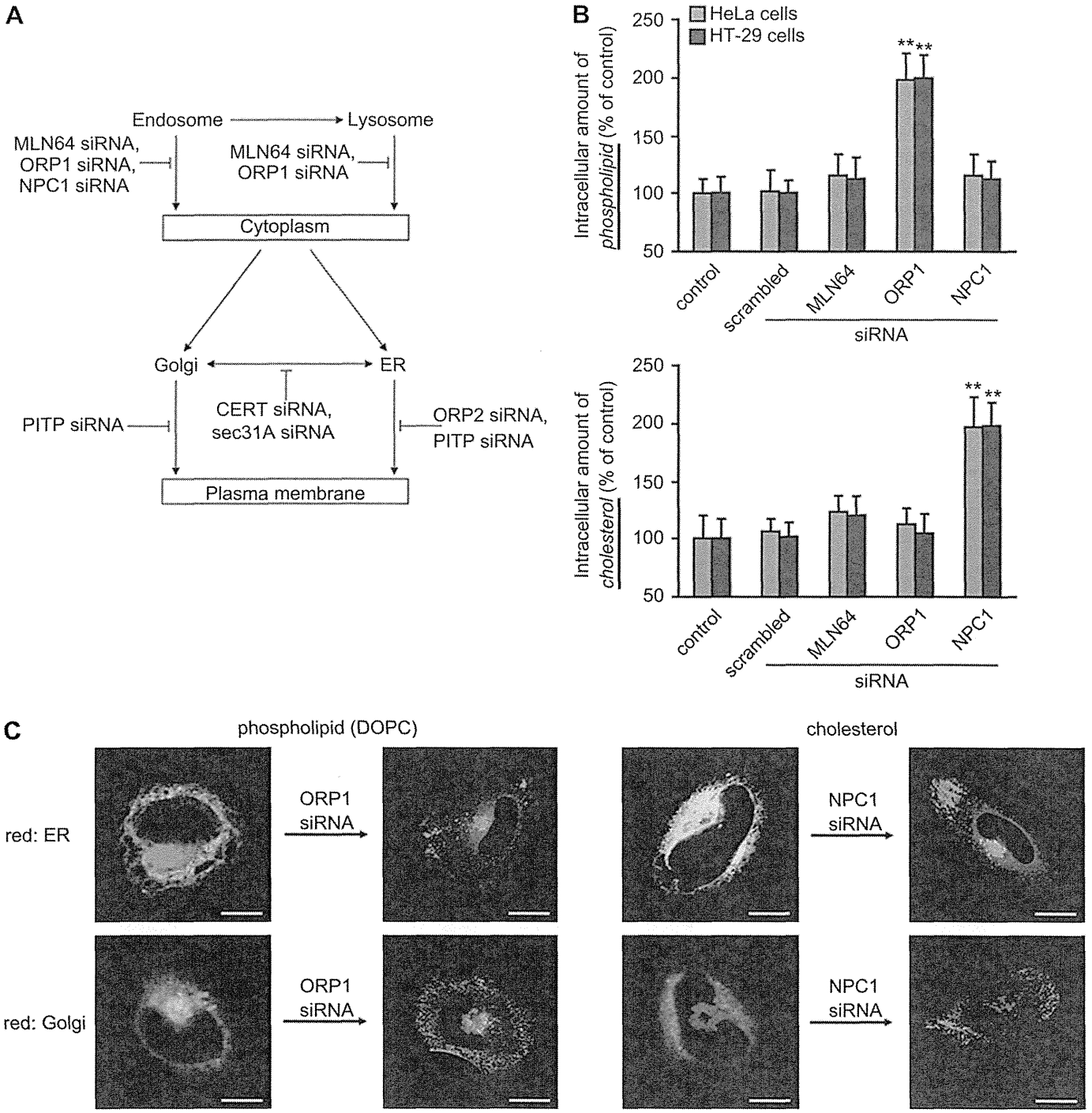

Fig. 3. Elucidation of escape mechanisms of DOPC/Chol from endosome/lysosome to the cytoplasm. (A) Schematic of proteins suppressed by siRNA. (B) The intracellular amounts of DOPC/Chol under suppressing conditions for each protein (MLN64, ORP1, and NPC1) at 24 h after addition of liposomes to HeLa and HT-29 cells. siRNAs against the targeted proteins were transfected using Lipofectamine RNAiMAX according to the recommended protocols. $^{**}P < 0.01$ compared with the corresponding control group. Each value represents the mean + SD ($n = 6$). (C) Confocal images showing the intracellular localization of DOPC/Chol under suppressing conditions for ORP1 or NPC1 at 24 h after the addition of liposomes to HeLa cells. Liposomes were labeled with NBD-labeled DOPC/Chol (green), and ER/Golgi apparatus were labeled with CellLight-RFP (red). Scale bars, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in transport from the endosome/lysosome to the cytoplasm, the intracellular amounts of DOPC derived from liposomes with Chol were only increased when ORP1 was suppressed. However, the intracellular amounts of DOPC derived from liposomes without Chol were increased when ORP1 or NPC1 were suppressed (Fig. 6B). When CERT or sec31A expression was suppressed, the increased amounts of intracellular DOPC derived from liposomes without Chol were lower than those derived from liposomes containing Chol (Fig. 6C). In addition, the intracellular amounts of DOPC obtained from liposomes without Chol were increased when PITP or ORP2 expression were knocked-down (Fig. 6D). With respect to extracellular efflux, although DOPC derived from liposomes with Chol were effluxed *via* ABCG1, DOPC derived from liposomes without Chol were effluxed *via* ABCA1 or ABCB1 (Fig. 6E).

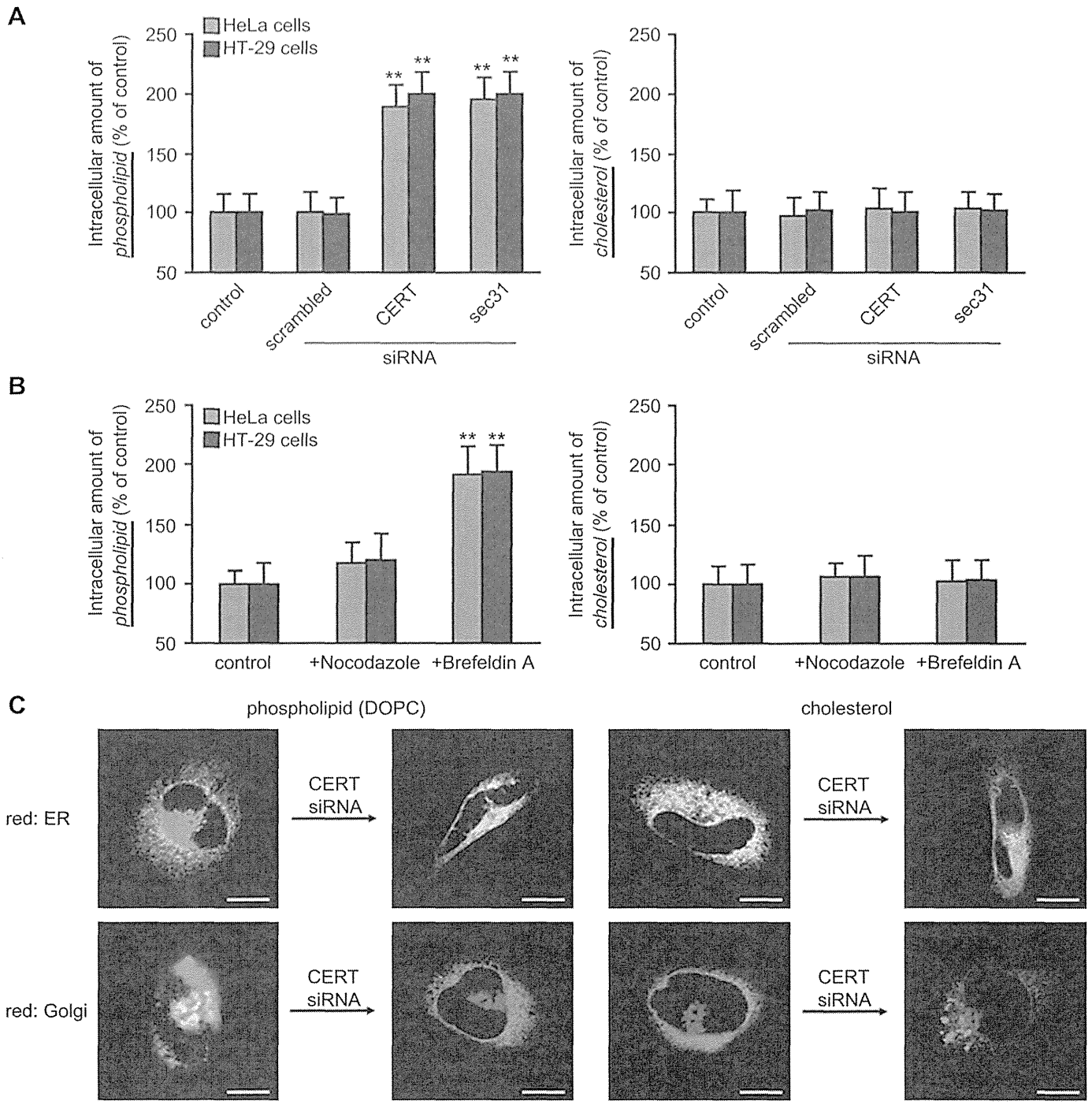

Fig. 4. Involvement of ER-Golgi transport in the intracellular trafficking of DOPC/Chol. (A) The intracellular amounts of DOPC/Chol under suppressing conditions for CERT or sec31A at 24 h after the addition of liposomes to HeLa and HT-29 cells. siRNAs against the targeted proteins were transfected using Lipofectamine RNAiMAX according to recommended protocols. $^{**}P < 0.01$ compared with the corresponding control group. Each value represents the mean + SD ($n = 6$). (B) The intracellular amounts of DOPC/Chol under Golgi destructive conditions at 24 h after the addition of liposomes in HeLa and HT-29 cells. The Golgi apparatus was destroyed following pre-incubation with brefeldin A (1.0 μg/mL) and nocodazole (30 μM) for 30 min $^{**}P < 0.01$ compared with the corresponding control group. Each value represents the mean + SD ($n = 6$). (C) Confocal images indicating the intracellular localization of DOPC/Chol under suppressing conditions for CERT at 24 h after the addition of liposomes to HeLa cells. Liposomes were labeled with NBD-labeled DOPC/Chol (green), and ER/Golgi apparatus were labeled with CellLight-RFP (red). Scale bars, 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The aim of this study was to elucidate the trafficking mechanisms involved in the intracellular transport and extracellular efflux of liposomal components. In the present study, we used liposomes containing NBD-labeled DOPC and Chol. As shown in Figs. 1B and 7, the intracellular uptake of DOPC and Chol, which are liposomal components, was inhibited in the presence of chlorpromazine in HeLa and HT-29 cells; suggesting that both components are taken up into the cells *via* clathrin-mediated endocytosis. Kheirolomoom and Ferrara reported that endogenous Chol is taken up by cells *via* caveolae-mediated endocytosis [12]. On the other

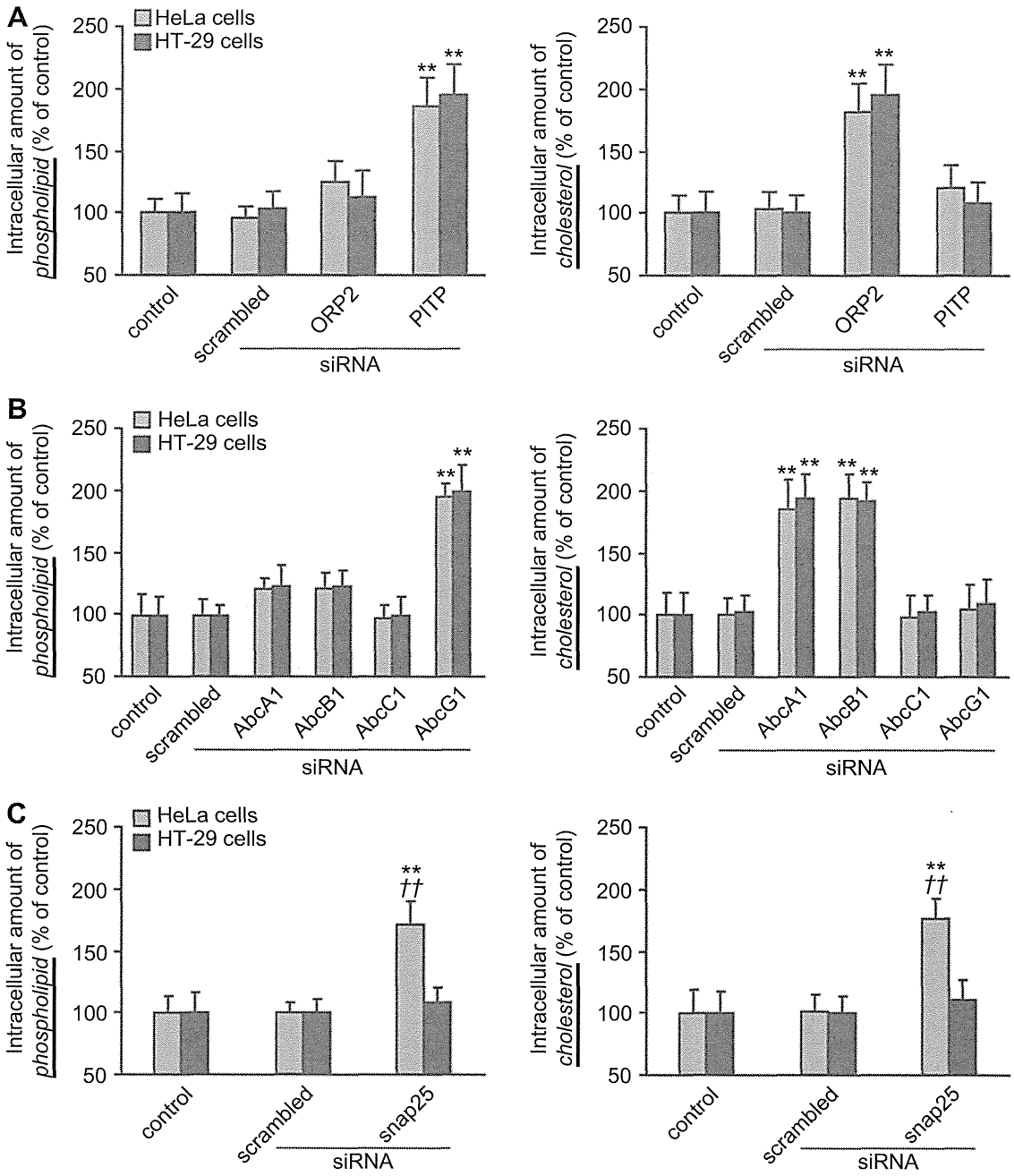

Fig. 5. Elucidation of the extracellular efflux mechanism of DOPC/Chol. (A) The intracellular amounts of DOPC/Chol under suppressing conditions for ORP2 or PITP at 24 h after the addition of liposomes to HeLa and HT-29 cells. (B) The intracellular amounts of DOPC/Chol under suppressing conditions for various ABC transporters (ABCA1, ABCB1, ABCC1, and ABCG1) at 24 h after addition of liposomes to HeLa and HT-29 cells. (C) The intracellular amounts of DOPC/Chol under suppressing conditions for snap-25 at 24 h after addition of liposomes to HeLa and HT-29 cells. siRNAs against the targeted proteins were transfected using Lipofectamine RNAiMAX according to recommended protocols. $^{**}P < 0.01$ compared with the corresponding control group. Each value represents the mean + SD ($n = 6$).

hand, most of liposomes are reported to be taken up into cells *via* clathrin-mediated endocytosis [8,9]. Our results would suggest that liposomes constructed with DOPC and Chol are taken up into cells *via* clathrin-mediated endocytosis when their structures are spherical according to our experimental conditions. With respect to intracellular localization of both components following intracellular uptake, DOPC was localized to the ER and Golgi apparatus, and Chol was localized to the ER (Fig. 2). This suggests that each liposomal component might be transported *via* different intracellular trafficking process after endocytosis. These findings indicate that the liposomes used in this study are degraded somewhere between the endosomes and the ER/Golgi apparatus.

We also investigated the intracellular trafficking mechanisms of DOPC and Chol. Under these conditions, when the extracellular efflux of liposomal components was decreased by suppressing specific intracellular transport processes, the intracellular amount of each liposomal component was increased. As shown in Fig. 3, the intracellular amounts of DOPC and Chol were increased, and the intracellular trafficking of DOPC and Chol to the ER or Golgi apparatus was suppressed, when expression of ORP1 and NPC1 was

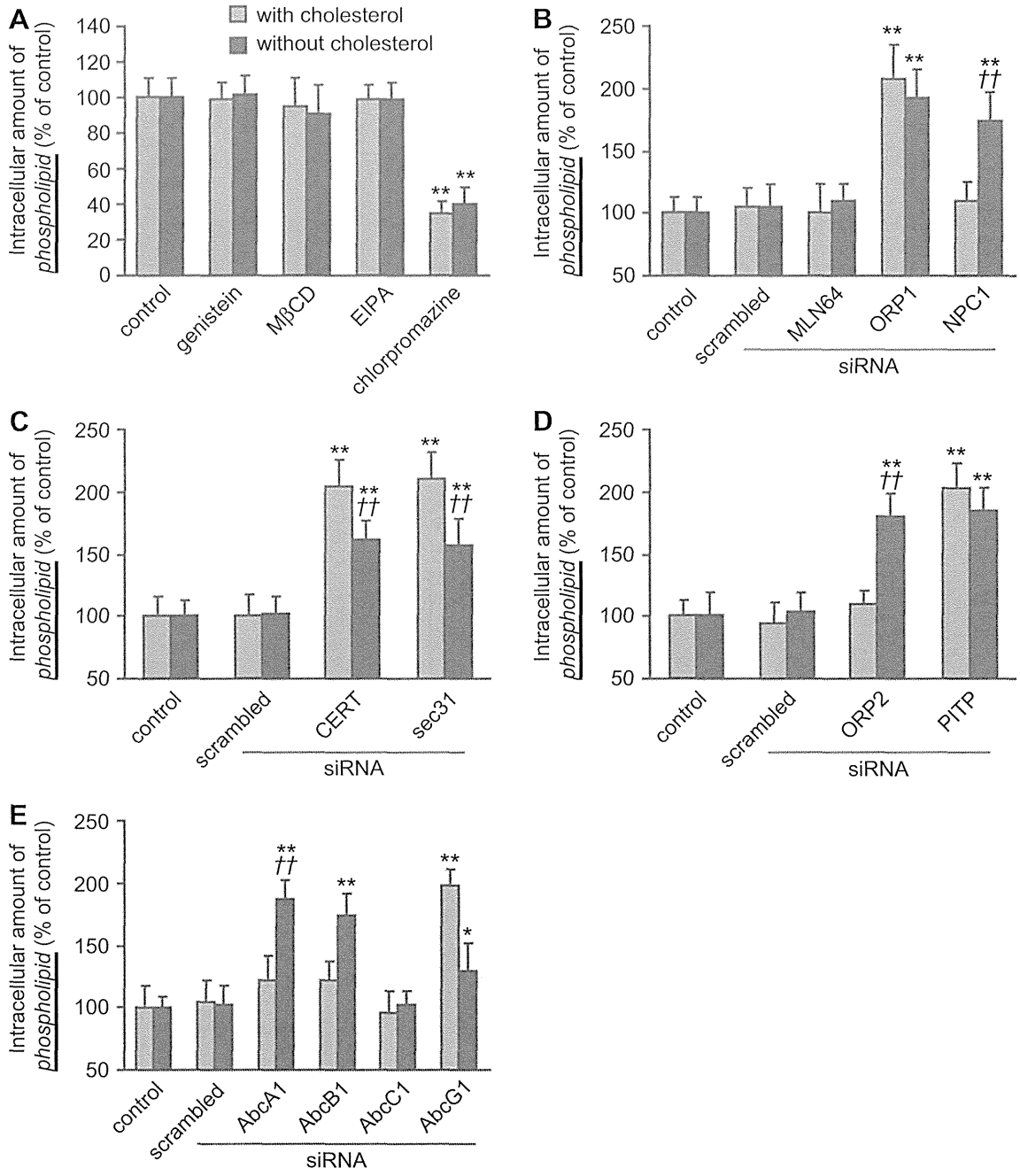

Fig. 6. Effects of cholesterol on intracellular trafficking of DOPC in HeLa cells. (A) The intracellular transport of DOPC at 2 h after addition of liposomes with or without Chol. Each endocytosis inhibitor was added to cells at 30 min before the addition of liposomes. (B) The intracellular amounts of DOPC under suppressing conditions for each protein (MLN64, ORP1, and NPC1) at 24 h after the addition of liposomes with or without Chol. (C) The intracellular amounts of DOPC under suppressing conditions for CERT or sec31A at 24 h after the addition of liposomes with or without Chol. (D) The intracellular amounts of DOPC under suppressing conditions for ORP2 or PITP at 24 h after the addition of liposomes with or without Chol. (E) The intracellular amounts of DOPC under suppressing conditions for various ABC transporters (ABCA1, ABCB1, ABCC1, and ABCG1) at 24 h after the addition of liposomes with or without Chol. siRNAs against each protein were transfected using Lipofectamine RNAiMAX according to recommended protocols. $^{**}P < 0.01$ compared with the corresponding control group. Each value represents the mean + SD ($n = 6$).

inhibited, respectively (Fig. 3C). Our results suggest that DOPC and Chol are transferred separately from the endosome/lysosome to the cytoplasm by ORP1 and NPC1, respectively (Fig. 7). Because NPC1 is only localized in the late endosomes [45,46], these findings suggest that liposomes, at least those used in this study, are degraded into their individual components in the late endosomes. Additionally, it has been previously reported that NPC1 directly transports lipophilic compounds into the cytoplasm, and that this does not occur *via* lysosomes [45,46]; therefore, it is believed that Chol is not transferred into lysosomes with various types of degrading enzymes. Recently, conjugation of Chol to various drugs has been studied for improved stability of certain physiological conditions [47,48]. The modification of Chol enables the evasion of transfer to lysosomes because of NPC1-mediated transport [45,46]. Therefore, Chol modification is suitable for the intracellular delivery of degradable compounds such as nucleic acids or proteins.

The intracellular trafficking of Chol derived from liposomes was not affected when ER-to-Golgi transport was suppressed (Fig. 4).

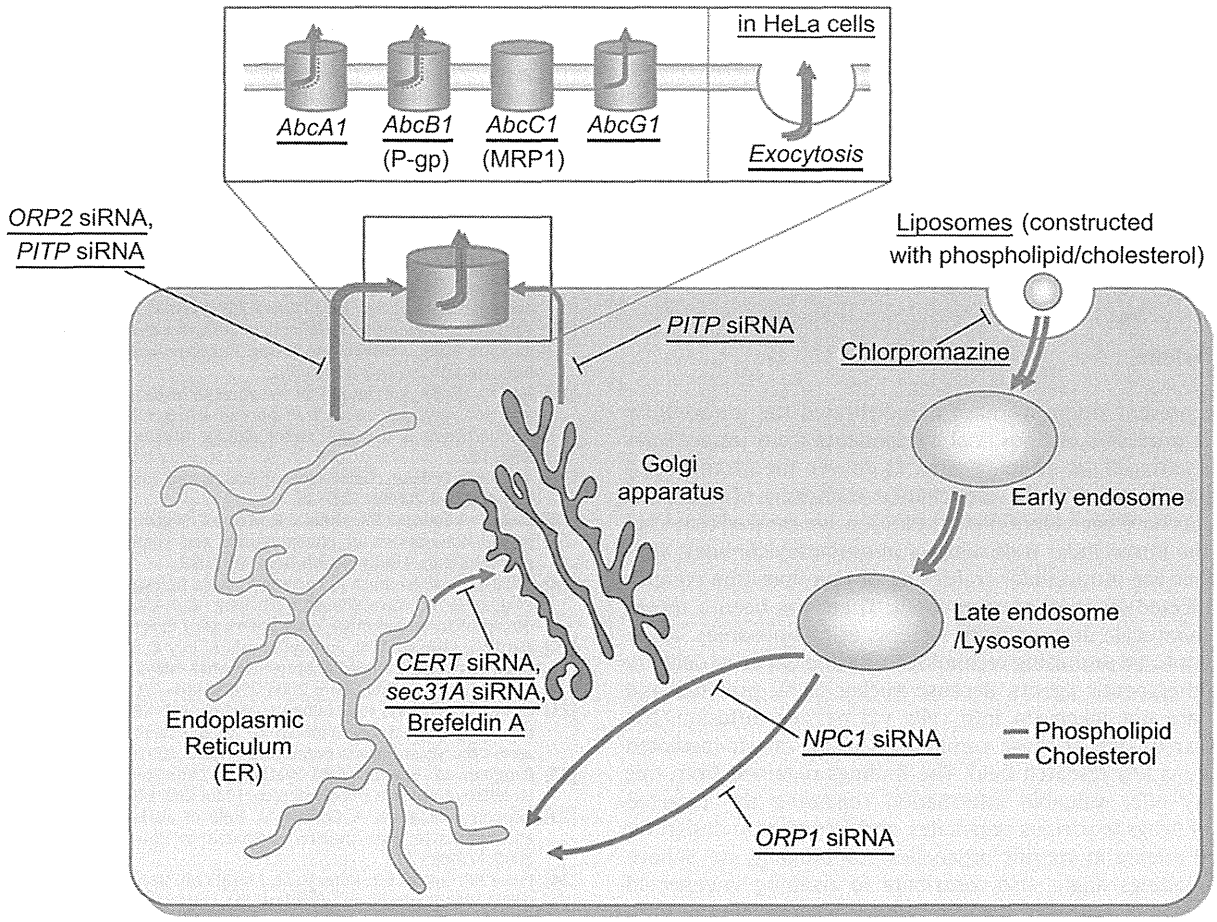

Fig. 7. Predicted mechanism of intracellular trafficking of DOPC and Chol derived from liposomes.

Our results also suggest that ER-to-Golgi transport is not involved in the intracellular trafficking of Chol (Fig. 7), corresponding with our results relating to the intracellular localization of Chol (Fig. 2). Some researchers have reported that endogenous Chol is transported to the ER but not *via* the Golgi apparatus following transfer to the cytoplasm [16,17]. These results may indicate that the synthesized liposomes containing Chol are transported along similar pathways as endogenous Chol. Because the amounts of intracellular DOPC were increased by CERT/sec31A suppression and Golgi destruction (Fig. 4), it is suggested that DOPC is partly transported to the Golgi apparatus *via* the ER (Fig. 7). It is known that CERT and sec31A are involved in the ER-to-Golgi transport of ceramide [35,36] and membrane-to-membrane transport of lipidic compounds [37,49]. DOPC possesses a similar molecular structure as ceramide; therefore, the intracellular trafficking of both compounds might be controlled by these proteins.

We investigated the extracellular efflux mechanisms of DOPC and Chol. As shown in Fig. 5A, the transport of DOPC and Chol derived from liposomes to the cell membrane was controlled by PITP and ORP2, respectively. It has been reported that PITP and ORP2 are involved in vesicle-independent intermembrane transport of lipophilic compounds [39–44], and that the ER and Golgi apparatus possess closed sites at the cell membrane [50]; therefore, DOPC and Chol might be transferred to the cell membrane *via* these closed sites. Following knockdown experiments of various ABC transporters, DOPC was observed to be mainly effluxed *via* ABCG1, and Chol was effluxed *via* ABCA1 and ABCB1 (Fig. 5B); indicating that both components are effluxed *via* different pathways (Fig. 7). It is known that ABCA1 is involved in phosphatidylcholine/Chol transport [51]. DOPC derived from DOPC/Chol liposomes were effluxed *via* ABCG1, not ABCA1 (Fig. 5B); suggesting that the affinity of Chol to ABCA1 is higher than that of DOPC. Additionally, Chol was also effluxed *via* ABCB1, also known as P-glycoprotein (P-gp) (Fig. 5B). It has been reported that anti-cancer drug resistance based on the over-expression of P-gp is overcome by Chol-containing liposomal formulations [52]. Therefore, the competitive inhibition by Chol against P-gp might be involved in overcoming anti-cancer drug resistance in one way or possibility.

We showed that the intracellular trafficking mechanisms of liposomal components are nearly identical in HeLa and HT-29 cells. However, the involvement of exocytosis in extracellular efflux of liposomal components was only observed in HeLa cells (Figs. 5C and 7). It has been reported that the extracellular efflux of various components, including proteins and lipids, *via* exocytosis is controlled by SNARE proteins such as snap-25 [53,54]. The expression levels of SNARE-related proteins are high in specific cancer cells, including HeLa cells [53–55]. Therefore, these cells might possess another extracellular efflux mechanism of liposomal components by constructing exocytic vesicles containing DOPC or Chol.

In conventional liposomal formulations, Chol is included as a helper lipid to control membrane fluidity and liposomal stability [56]. In the present study, we also investigated the effects of Chol on intracellular trafficking of DOPC derived from liposomes. As shown in Fig. 6, the intracellular trafficking of DOPC derived from liposomes without Chol was partly controlled by NPC1, ORP2, and

ABCA1, which are involved in the intracellular trafficking of Chol. The involvement of CERT/sec31A controlling ER-to-Golgi transport was decreased in the intracellular trafficking of DOPC derived from liposomes without Chol (Fig. 6C). These findings suggest that the intracellular trafficking of DOPC is affected by Chol-containing liposomes. The intracellular transport of DOPC is partly controlled by proteins involved in Chol trafficking; however, the affinity of Chol for each protein is assumed to be higher than that observed for DOPC. Therefore, it is considered that the intracellular trafficking of DOPC might be affected by Chol when it is one of the liposomal components.

5. Conclusions

In the present study, we have demonstrated the intracellular trafficking processes of liposomal components from intracellular uptake to extracellular efflux *in vitro*. Moreover, the proteins and transporters involved in the intracellular trafficking of liposomal components have been identified. In addition, we showed that Chol affected the intracellular trafficking of phosphatidylcholines; suggesting that the intracellular trafficking of one liposome component is affected by another component. There has been a recent focus on nanoscale drug delivery systems using liposomes. These are thought to be promising technologies for the effective delivery of chemotherapeutic agents. Because nucleic acids, proteins, and peptides are not taken up into cells *via* passive diffusion, the intracellular uptake of these compounds using carrier-mediated technology is key research field. The findings obtained from this study may offer valuable information regarding the targeted delivery of drugs to various organelles, and formulation design for controlled release in specific organelles. Additionally, we believe that our findings might also contribute to avoiding unexpected toxic side-effects following the intracellular accumulation of liposomal components.

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